

β -FRUCTOFURANOSIDASE AND ITS GENE, METHOD OF
ISOLATING β -FRUCTOFURANOSIDASE GENE, SYSTEM FOR
PRODUCING β -FRUCTOFURANOSIDASE, AND
 β -FRUCTOFURANOSIDASE VARIANT

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Background of the Invention

Field of the Invention

The present invention relates to a β -fructofuranosidase gene, a process for isolating the gene, and a system for producing a β -fructofuranosidase. More particularly, the present invention relates to a novel β -fructofuranosidase, a DNA encoding it, and a process for isolating a DNA encoding β -fructofuranosidase; a novel mold fungus having no β -fructofuranosidase and a process for producing a recombinant β -fructofuranosidase using the mold fungus as a host; and a β -fructofuranosidase variant which selectively and efficiently produces a specific fructooligosaccharide such as 1-kestose from sucrose.

Background Art

The molecular structure of a fructooligosaccharide is the same as that of sucrose, except that the fructose half of a fructooligosaccharide is coupled with another one to three fructose molecules at positions C1 and C2 via a β bond. Fructooligosaccharides are indigestible sugars known for their physiological advantages, such as the facilitation of Bifidobacterial growth in the intestines, metabolic stimulation for cholesterol and other lipids, and little cariosity.

Fructooligosaccharides are found in plants, such as asparagus, onion, Jerusalem-artichoke and honey. They are also synthesized from sucrose by the newly industrialized mass production technique using fructosyltransfer reaction which is catalyzed by a β -fructofuranosidase derived from a microorganism. However, as β -fructofuranosidase preparations which are currently used for the industrial production of fructooligosaccharides is a cell-bound β -fructofuranosidase derived from *Aspergillus niger*, they contain a relatively large proportion of proteins as impurities. Therefore, a need still exists for a high-purity β -fructofuranosidase preparation with little unwanted proteins and a high titer. Further, an

extracellular β -fructofuranosidase is desired in an attempt to improve efficiently by using it in a fixed form, as an extracellularly available enzyme is more suitable for fixation.

- Genes encoding β -fructofuranosidase have been isolated from
- 5 bacteria (Fouet, A., *Gene*, 45, 221-225 (1986), Martin, I. et al., *Mol. Gen. Genet.*, 208, 177-184 (1987), Steinintz, M. et al., *Mol. Gen. Genet.*, 191, 138-144 (1983), Scholle, R. et al., *Gene*, 80, 49-56 (1989), Aslanidis, C. et al., *J. Bacteriol.*, 171, 6753-6763 (1989), Sato, Y. and Kuramitsu, H. K., *Infect. Immun.*, 56, 1956-1960 (1989),
 - 10 Gunasekaran, P. et al., *J. Bacteriol.*, 172, 6727-6735 (1990)); yeast (Taussing, R. and M. Carlson, *Nucleic Acids Res.*, 11, 1943-1954 (1983), Laloux, O. et al., *FEBS Lett.*, 289, 64-68 (1991); mold (Boddy, L. M. et al., *Curr. Genet.*, 24, 60-66 (1993); and plants (Arai, M. et al., *Plant Cell Physiol.*, 33, 245-252 (1992), Unger, C. et al. *Plant Physiol.*,
 - 15 104, 1351-1357 (1994), Elliott, K. et al., *Plant Mol. Biol.*, 21, 515-524 (1993), Sturm, A. and Chrispeels, M. J., *Plant Cell*, 2, 1107-1119 (1990)). However, to the best knowledge of the inventors, no gene has been found which encodes a β -fructofuranosidase having transferase activity and is usable for the industrial production of
 - 20 fructooligosaccharides.

- If a β -fructofuranosidase gene usable for the industrial production of fructooligosaccharides is obtained, other functionally similar genes may be isolated, making use of their homology to the former. To the best knowledge of the inventors, no case has been
- 25 reported on the screening of a new β -fructofuranosidase gene using this technique. A process for isolating a β -fructofuranosidase gene by this approach may also be applied to the screening of β -fructofuranosidase enzyme to achieve significantly less effort and time than in conventional processes: first, using a
 - 30 β -fructofuranosidase gene as a probe, a similar β -fructofuranosidase gene is isolated, making use of its homology to the former; then, the isolated gene is introduced and expressed in a host which does not metabolize sucrose, such as *Trichoderma viride*, or a mutant yeast which lacks sucrose metabolizing capability (Oda, Y.
 - 35 and Ouchi, K., *Appl. Environ. Microbiol.*, 1989, 55, 1742-1747); a homogeneous preparation of β -fructofuranosidase is thus obtained as a genetic product with significantly less effort and time of screening.

Furthermore, if the resultant β -fructofuranosidase exhibits desirable characteristics, its encoding gene may be introduced in a safe and highly productive strain to enable the production of the desired β -fructofuranosidase.

- 5 In addition, for producing such desirable β -fructofuranosidase, designing a system for production, particularly a host which does not metabolize sucrose, is an important consideration. Using a host which intrinsically has β -fructofuranosidase activity would result in a mixture of the endogenous β -fructofuranosidase of the host and
10 the β -fructofuranosidase derived from the introduced gene. In this case, to take advantage of the β -fructofuranosidase derived from the introduced gene, it must be isolated from the endogenous β -fructofuranosidase of the host before application. On the contrary, using a host which lacks β -fructofuranosidase activity would
15 eliminate the need for enzyme isolation. In other words, the resultant unpurified enzyme would show the desirable characteristics of the β -fructofuranosidase derived from the introduced gene. Known examples of microorganisms which do not have β -fructofuranosidase activity include the *Trichoderma* strains and
20 yeast mutants lacking sucrose metabolizing capability (Oda, Y. Ibid.) as described above. However, considering that the resultant β -fructofuranosidase will be applied in food industry, a better candidate for a host would be a strain having no β -fructofuranosidase selected from *Aspergillus* mold fungi which
25 have been time-tested for safety through application to foods and industrial production of enzymes.

- Furthermore, if a β -fructofuranosidase gene usable for the industrial production of fructooligosaccharides is obtained, it may enable the development of a mutant with improved characteristics.
30 For example, β -fructofuranosidase which produces 1-kestose selectively and efficiently would provide the following advantage:

- The molecular structures of 1-kestose and nystose, which make up part industrially produced fructooligosaccharide mixtures of today, are the same as that of sucrose except that their fructose half is
35 coupled with one and two molecules of fructose, respectively. It has been found recently that their high-purity crystals exhibit new desirable characteristics both in physical properties and food

processing purpose while maintaining the general physiological advantages of fructooligosaccharides (Japanese Patent Application No. 222923/1995, Japanese Patent Laid-Open Publication No. 31160/1994). In this sense, they are fructooligosaccharide preparations having new features.

- In consideration of the above, some of the inventors have proposed an industrial process for producing crystal 1-kestose from sucrose (Japanese Patent Application No. 64682/1996, Japanese Patent Application No. 77534/1996, and Japanese Patent Application No. 77539/1996). According to this process, a β -fructofuranosidase harboring fructosyltransferase activity is first allowed to act on sucrose to produce 1-kestose; the resultant 1-kestose is fractionated to a purity of 80% or higher by chromatographic separation; then, using this fraction as a crystallizing sample, crystal 1-kestose is obtained at a purity of 95% or higher. The β -fructofuranosidase harboring fructosyltransferase activity used in this process should be able to produce 1-kestose from sucrose at a high yield while minimizing the byproduct nystose, which inhibits the reactions in the above steps of chromatographic separation and crystallization. In the enzyme derived from *Aspergillus niger*, which is currently used for the industrial production of fructooligosaccharide mixtures, the 1-kestose yield from sucrose is approximately 44%, while 7% is turned to nystose (Japanese Patent Application No. 64682/1996). These figures suggest that the enzyme has room for improvement in view of the industrial production of crystal 1-kestose. As a next step, new enzymes having more favorable characteristics were successfully screened from *Penicillium roqueforti* and *Scopulariopsis brevicaulis*. These enzymes were able to turn 47% and 55% of sucrose into 1-kestose, respectively, and 7% and 4% to nystose (Japanese Patent Application No. 77534/1996, and Japanese Patent Application No. 77539/1996). Although these figures show that the new enzymes were superior to the enzyme derived from *Aspergillus niger* for higher 1-kestose yields and less nystose production from sucrose, the productivity and stability of the enzymes were yet to be improved. Thus, it is awaited to see a new enzyme that maintains the productivity and stability of the enzyme derived from *Aspergillus niger*,

which is currently used for the industrial production of fructooligosaccharide mixtures, while achieving a sucrose-to-1-kestose yield comparable or superior to that of the enzymes derived from *Penicillium roqueforti* and *Scopulariopsis brevicaulis*.

Summary of the Invention

The inventors have now successfully isolated a novel β -fructofuranosidase gene, and developed a process for isolating other β -fructofuranosidase genes using the novel gene.

The inventors have also successfully produced a novel mold fungus having no β -fructofuranosidase activity, and developed a system for producing a recombinant β -fructofuranosidase using the mold fungus as a host.

Further, the inventors have found that the characteristics of β -fructofuranosidase with fructosyltransferase activity change with its amino acid sequence, and have successfully produced a β -fructofuranosidase variant which selectively and efficiently produces a specific fructooligosaccharide such as 1-kestose from sucrose.

The present invention is based on these findings.

Thus, the first aspect of the present invention provides a novel β -fructofuranosidase gene and a β -fructofuranosidase encoded by the gene.

The second aspect of the present invention provides a process for isolating a β -fructofuranosidase gene using the novel β -fructofuranosidase gene. The process according to the second aspect of the present invention also provides a novel β -fructofuranosidase.

In addition, the third aspect of the present invention provides a novel mold fungus having no β -fructofuranosidase activity and a system for producing a recombinant β -fructofuranosidase using the mold fungus as a host.

Further, the fourth aspect of the present invention provides a β -fructofuranosidase variant which selectively and efficiently produces a specific fructooligosaccharide such as 1-kestose from sucrose.

The β -fructofuranosidase according to the first aspect of the present invention has the amino acid sequence of SEQ ID No. 1 as shown in the sequence listing.

In addition, the β -fructofuranosidase gene according to the first aspect of the present invention encodes the amino acid sequence of SEQ ID No. 1 as shown in the sequence listing.

Further, the process for isolating a β -fructofuranosidase gene according to the second aspect of the present invention is a process for isolating a β -fructofuranosidase gene, making use of its homology to a nucleotide sequence comprising all or part of the nucleotide sequence of SEQ ID No. 2 as shown in the sequence listing.

In addition, a novel β -fructofuranosidase which has been isolated in the process according to the second aspect of the present invention is a polypeptide comprising the amino acid sequence of SEQ ID No. 11 or 13 as shown in the sequence listing or a homologue thereof.

Furthermore, the mold fungus according to the third aspect of the present invention is a mold fungus having no β -fructofuranosidase by deleting all or part of the β -fructofuranosidase gene on the chromosome DNA of the original *Aspergillus* mold fungus.

The β -fructofuranosidase variant according to the fourth aspect of the present invention is a mutant β -fructofuranosidase with fructosyltransferase activity obtained by a mutation in the original β -fructofuranosidase thereof, wherein the variant comprises an insertion, substitution or deletion of one or more amino acids in, or an addition to either or both of the terminals of, the amino acid sequence of the original β -fructofuranosidase, and the composition of the fructooligosaccharide mixture produced from sucrose as a result of fructosyltransfer reaction by the β -fructofuranosidase variant differs from the composition of the fructooligosaccharide mixture produced by the original β -fructofuranosidase.

Brief Description of the Drawings

Figure 1 shows expression vector pAW20-Hyg in which the β -fructofuranosidase gene according to the present invention has been introduced.

Figure 2 shows expression vector pPRS01-Hyg in which a β -fructofuranosidase gene isolated in the process according to the second aspect of the present invention has been introduced.

Figure 3 is the restriction map of a DNA fragment comprising the *niaD* gene which has been derived from the *Aspergillus niger* NRRL4337.

Figure 4 shows the construction of plasmid pAN203.

Figure 5 shows the construction of plasmid pAN572.

Figure 6 is the restriction map of plasmid pAN120.

Figure 7 shows the construction of plasmid pY2831.

Figure 8 shows the construction of plasmid pYSUC (F170W).

Figure 9 shows the construction of plasmid pAN531.

Detailed Description of the Invention

Deposit of Microorganism

The novel mold fungus *Aspergillus niger* NIA1602 having no β -fructofuranosidase according to the present invention has been deposited in the National Institute of Bioscience and Human-Technology, Ministry of International Trade and Industry of Japan (Higashi 1-1-3, Tsukuba City, Ibaraki Pref., Japan) as of March 6, 1997, under Accession No. FERM-BP5853.

β -Fructofuranosidase according to the first aspect of the present invention

The polypeptide according to the first aspect of the present invention comprises the amino acid sequence of SEQ ID No. 1 as shown in the sequence listing. This polypeptide having the amino acid sequence of SEQ ID No. 1 has enzymatic activity as β -fructofuranosidase. The polypeptide according to the present invention involves a homologue of the amino acid sequence of SEQ ID No. 1 as shown in the sequence listing. The term "homologue" refers to an amino acid sequence in which one or more amino acids are inserted, substituted or deleted in, or added to either or both of the terminals of, the amino acid sequence of SEQ ID No. 1, while retaining β -fructofuranosidase activity. Such a homologue can be selected and produced by those skilled in the art without undue experiments by referring to the sequence of SEQ ID No. 1.

The β -fructofuranosidase having the amino acid sequence of

SEQ ID No. 1 has a high fructosyltransferase activity and efficiently produces fructooligosaccharides. Specifically, when a sucrose solution at a concentration of 10 wt% or more is used as a substrate for reaction, the fructosyltransferase activity is at least 10 times higher than hydrolytic activity, with 50% or more changed to fructooligosaccharides.

Gene encoding β -fructofuranosidase according to the first aspect of the present invention

The first aspect of the present invention provides, as a novel β -fructofuranosidase gene, a DNA fragment which comprises the nucleotide sequence encoding the amino acid sequence of SEQ ID No. 1.

A preferred embodiment of the present invention provides, as a preferred example of novel gene according to the present invention, a DNA fragment comprising the nucleotide sequence of SEQ ID No. 2 as shown in the sequence listing.

Generally, a nucleotide sequence which encodes the amino acid sequence of a given protein can be easily determined from the reference chart known as the "codon table." A variety of nucleotide sequences are available from those encoding the amino acid sequence of SEQ ID No. 1. Therefore, the term "a nucleotide sequence encoding the amino acid sequence of SEQ ID No. 1" refers to the meaning including the nucleotide sequence of SEQ ID No. 2, as well as nucleotide sequences which consist of the same codons as above allowing for degeneracy and encode the amino acid sequence of SEQ ID No. 1.

As described above, the present invention encompasses a homologue of the amino acid sequence of SEQ ID No. 1. Therefore, the DNA fragment according to the present invention involves a nucleotide sequence which encodes such a homologue.

As the nucleotide sequence of the DNA fragment according to the present invention is known, the DNA fragment may be obtained according to the procedure for the synthesis of a nucleic acid.

This sequence can be also obtained from *Aspergillus niger*, preferably *Aspergillus niger* ACE-2-1 (FERM-P5886 or ATCC20611), according to the procedure of genetic engineering. The specific process is described in more details later in Example A.

Expression of β -Fructofuranosidase Gene

The β -fructofuranosidase according to the first aspect of the present invention can be produced in a host cell which has been transformed by a DNA fragment encoding the enzyme. More specifically, a DNA fragment encoding the β -fructofuranosidase according to the first aspect of the present invention is introduced in a host cell in the form of a DNA molecule which is replicatable in the host cell and can express the above gene, particularly an expression vector, in order to transform the host cell. Then, the obtained transformant is cultivated.

Therefore, the present invention provides a DNA molecule which comprises a gene encoding the β -fructofuranosidase according to the present invention, particularly an expression vector. This DNA molecule is obtained by introducing a DNA fragment encoding the β -fructofuranosidase according to the present invention in a vector molecule. According to a preferred embodiment of the present invention, the vector is a plasmid.

The DNA molecule according to the present invention may be prepared by the standard technique of genetic engineering.

The vector applicable in the present invention can be selected as appropriate from viruses, plasmids, cosmid vectors, etc., considering the type of the host cell used. For example, a bacteriophage in the λ phage group or a plasmid in the pBR or pUC group may be used for *E. coli* host cells, a plasmid in the pUB group for *Bacillus subtilis*, and a vector in the YEp or YCp group for yeast.

It is preferable that the plasmid contain a selectable marker to ensure the selection of the obtained transformance, such as a drug-resistance marker or marker gene complementing an auxotrophic mutation. Preferred examples of marker genes include ampicillin-resistance gene, kanamycin-resistance gene, and tetracycline-resistance gene for bacterium host cells; N-(5'-phosphoribosyl)-anthranilate isomerase gene (TRP1), orotidine-5'-phosphate decarboxylase gene (URA3), and β -isopropylmalate dehydrogenase gene (LEU2) for yeast; and hygromycin-resistance gene (hph), bialaphos-resistance gene (Bar), and nitrate reductase gene (niaD) for mold.

It is also preferable that the DNA molecule for use as an

expression vector according to the present invention contain nucleotide sequences necessary for the expression of the β -fructofuranosidase gene, including transcription and translation control signals, such as a promoter, a transcription initiation signal, a
5 ribosome binding site, a translation termination signal, and a transcription termination signal.

Examples of preferred promoters include, in addition to the promoter on the inserted fragment which is able to function in the host, promoters such as those of lactose operon (lac), and tryptophan
10 operon (trp) for *E. coli*; promoters such as those of alcohol dehydrogenase gene (ADH), acid phosphatase gene (PHO), galactose regulated gene (GAL), and glyceraldehyde-3-phosphate dehydrogenase gene (GPD) for yeast; and promoters such as those of α -amylase gene (amy) and cellobiohydrolase I gene (CBHI) for mold.

15 When the host cell is *Bacillus subtilis*, yeast or mold, it is also advantageous to use a secretion vector to allow it to extracellularly secrete the produced recombinant β -fructofuranosidase. Any host cell with an established host-vector system may be used, preferably yeast, mold, etc. It is preferable also to use the mold fungus
20 according to the third aspect of the present invention to be described later.

A novel recombinant enzyme produced by the transformant described above is obtained by the following procedure: first, the host cell described above is cultivated under suitable conditions to obtain
25 the supernatant or cell bodies from the resultant culture, using a known technique such as centrifugation; cell bodies should be further suspended in a suitable buffer solution, then homogenized by freeze-and-thaw, ultrasonic treatment, or mortar, followed by centrifugation or filtration to separate a cell body extract containing
30 the novel recombinant enzyme.

The enzyme can be purified by combining the standard techniques for separation and purification. Examples of such techniques include processes such as heat treatment, which rely on the difference in thermal resistance; processes such as salt
35 sedimentation and solvent sedimentation, which rely on the difference in solubility; processes such as dialysis, ultrafiltration and gel filtration, and SDS-polyacrylamide gel electrophoresis, which rely on

the difference in molecular weight; processes such as ion exchange chromatography, which rely on the difference in electric charge; processes such as affinity chromatography, which rely on specific affinity; processes such as hydrophobic chromatography and reversed-phase partition chromatography, which rely on the difference in hydrophobicity; and processes such as isoelectric focusing, which rely on the difference in isoelectric point.

Production of fructooligosaccharides using the β -fructofuranosidase according to the first aspect of the present invention

The present invention further provides a process for producing fructooligosaccharide using the recombinant host or recombinant β -fructofuranosidase described above.

In the process for producing fructooligosaccharides according to the present invention, the recombinant host or recombinant β -fructofuranosidase described above is brought into contact with sucrose.

The mode and conditions where the recombinant host or recombinant β -fructofuranosidase according to the present invention comes in contact with sucrose are not limited in any way provided that the novel recombinant enzyme is able to act on the sugar. A preferred embodiment for contact in solution is as follows: The sucrose concentration may be selected as appropriate in the range where the substrate sugar can be dissolved. However, considering the conditions such as the specific activity of the enzyme and reaction temperature, the concentration should generally fall in the range of 5 to 80%, preferably 30 to 70%. The temperature and pH for the reaction of the sugar by the enzyme should preferably be optimized for the characteristics of the novel recombinant enzyme. Therefore, the reasonable conditions are about 30 to 80 °C, pH 4 to 10, preferably 40 to 70 °C, pH 5 to 7.

The degree of purification of the novel recombinant enzyme may be selected as appropriate. The enzyme may be used either as unpurified in the form of supernatant from a transformant culture or cell body homogenate, as purified after processed in various purification steps, or as isolated after processed by various purification means.

Furthermore, the enzyme may be brought into contact with sucrose as fixed on a carrier using the standard technique.

- The fructooligosaccharides thus produced is purified from the resulting solution according to a known procedure. For example, the solution may be heated to deactivate the enzyme, decolorized using activated carbon, then desalted using ion exchange resin.

Process for isolating a β -fructofuranosidase gene according to the second aspect of the present invention

- In the process for isolating a gene according to the second aspect of the present invention, the nucleotide sequence of SEQ ID No. 2 is used.

- The process for isolating a gene according to the second aspect of the present invention makes use of its homology to a nucleotide sequence comprising all or part of the nucleotide sequence of SEQ ID No. 2 as shown in the sequence listing. Examples of such processes include:

- a) screening a gene library which presumably contains a β -fructofuranosidase gene using the nucleotide sequence as a probe.
- b) preparing a primer based on the nucleotide sequence information, then performing PCR using a sample which presumably contains a β -fructofuranosidase gene as a template.

More specifically, process a) above comprises:

- preparing a gene library which presumably contains a β -fructofuranosidase gene;
- screening the gene library using a nucleotide sequence comprising all or part of the nucleotide sequence of SEQ ID No. 2 as shown in the sequence listing to select sequences which hybridize with the nucleotide sequence comprising all or part of the nucleotide sequence of SEQ ID No. 2 as shown in the sequence listing from the gene library, then isolating the selected sequences, and
- isolating a β -fructofuranosidase gene from the sequences which have been selected and isolated from the gene library.

The gene library may be a genomic DNA library or a cDNA library, and may be prepared according to a known procedure.

- It is preferable that the nucleotide sequence comprising all or part of the nucleotide sequence of SEQ ID No. 2 for use in screening the gene library be a nucleotide sequence comprising part of the

nucleotide sequence of SEQ ID No. 2, or a probe. Preferably, the probe should be marked.

5 The procedures for screening the gene library, marking the probe, isolating the marked and selected sequences, and further isolating a β -fructofuranosidase gene from the isolated sequences may be performed according to the standard techniques of genetic engineering under suitably selected conditions. Those skilled in the art would be able to select these procedures and conditions easily by referring to the sequence of SEQ ID No. 2.

10 On the other hand, process b) above comprises:

preparing a primer consisting of a nucleotide sequence which comprises all or part of the nucleotide sequence of SEQ ID No. 2 as shown in the sequence listing,

15 carrying out PCR process on the primer using a sample which presumably contains a β -fructofuranosidase gene as a template, and isolating a β -fructofuranosidase gene from the amplified PCR product.

The procedures for preparing the primer to be used, for preparing a sample which presumably contains a β -fructofuranosidase gene, and for PCR may be performed according to the standard techniques of genetic engineering under suitably selected conditions. Those skilled in the art would be able to select these procedures and conditions easily by referring to the sequence of SEQ ID No. 2.

25 The scope of application of the process for isolating a β -fructofuranosidase gene according to the present invention is not limited in any way provided that β -fructofuranosidase is presumably contained, such as Eumycetes, specifically Aspergillus, Penicillium or Scopulariopsis microorganisms.

30 Novel β -fructofuranosidase and gene encoding same obtained by the second aspect of the present invention.

The process for isolating a gene according to the second aspect of the present invention provides a novel β -fructofuranosidase enzyme having the amino acid sequence of SEQ ID No. 11 or 13 as shown in the sequence listing.

The β -fructofuranosidase enzyme according to the present invention may be a homologue of the amino acid sequence of SEQ ID

No. 11 or 13 as shown in the sequence listing. The term "homologue" refers to an amino acid sequence in which one or more amino acids are inserted, substituted or deleted in, or added to either or both of the terminals of, the amino acid sequence of SEQ ID No. 11 or 13, while retaining β -fructofuranosidase activity. Such a homologue can be selected and produced by those skilled in the art without undue experiments by referring to the sequence of SEQ ID No. 11 or 13.

The β -fructofuranosidase having the amino acid sequence of SEQ ID No. 11 or 13 has a high fructosyltransferase activity and efficiently produces fructooligosaccharides. Specifically, when a sucrose solution at a concentration of 30 % or more is used as a substrate for reaction, the fructosyltransferase activity is at least 4 times and 7 times higher, respectively, than hydrolytic activity, with 50% or more changed to fructooligosaccharides.

The novel β -fructofuranosidase gene provided by the process for isolating a gene according to the second aspect of the present invention comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID No. 11 or 13 as shown in the sequence listing or a homologue thereof.

Generally, a nucleotide sequence which encodes the amino acid sequence of a given protein can be easily determined from the reference chart known as the "codon table." Then, a variety of nucleotide sequences are available from those encoding the amino acid sequence of SEQ ID No. 11 or 13. Therefore, the term "a nucleotide sequence encoding the amino acid sequence of SEQ ID No. 11 or 13" refers to the meaning including the nucleotide sequence of SEQ ID No. 12 or 14, as well as nucleotide sequences which consist of the same codons as above allowing for degeneracy and encode the amino acid sequence of SEQ ID No. 11 or 13.

A preferred embodiment of the present invention provides a DNA fragment comprising the nucleotide sequence of SEQ ID No. 12 or 14 as shown in the sequence listing as preferred examples of the novel gene according to the present invention.

As described above, the enzyme encoded by the novel gene according to the present invention involves a homologue of the amino acid sequence of SEQ ID No. 11 or 13. Therefore, the DNA fragment according to the present invention may be a nucleotide sequence

which encodes such a homologue.

As the nucleotide sequence is known for the DNA fragment according to the present invention, the DNA fragment may be obtained according to procedure for the synthesis of a nucleic acid.

5 The sequence can be obtained from *Penicillium roqueforti* or *Scopulariopsis brevicaulis*, preferably *Penicillium roqueforti* IAM7254 or *Scopulariopsis brevicaulis* IFO4843, using the procedures of genetic engineering. The specific process is described in more details later in Example B.

10 Aspergillus mold fungus having no β -fructofuranosidase according to the third aspect of the present invention and preparation thereof

An Aspergillus mold fungus having no β -fructofuranosidase according to the third aspect of the present invention refers to an
15 Aspergillus mold fungus whose culture's supernatant and/or cell body homogenate provides unpurified enzyme which, when allowed to react with sucrose, does not change the substrate sucrose.

Such a mold fungus is obtained by deactivating a β -fructofuranosidase gene, deactivating the mechanism involved in the expression of a β -fructofuranosidase gene, or deactivating the
20 mechanism involved in the synthesis and secretion of the β -fructofuranosidase protein.

However, it is preferable that the β -fructofuranosidase gene itself be deactivated, in view of the stability of mutation and the
25 productivity of enzyme. It is especially preferable that all or part of the region encoding β -fructofuranosidase be deleted.

Available procedures for preparing such a mold fungus include the use of a mutagen such as NTG (1-methyl-3-nitro-1-nitrosoguanidine) or ultraviolet rays to induce
30 mutation in the original Aspergillus mold fungus. However, a process using the DNA recombination technology is preferred.

Examples of procedures for deactivating a β -fructofuranosidase gene using DNA recombination technology include methods using homologous recombination, which are
35 subdivided into two types of methods: one-step gene targeting and two-step gene targeting.

In one-step gene targeting, an insertion vector or substitution

vector is used.

As an insertion vector, a vector bearing a deactivated β -fructofuranosidase gene and a selectable marker gene for selecting the transformants is prepared. The deactivated β -fructofuranosidase gene is the same as the original β -fructofuranosidase gene except that it contains two discrete mutations (preferably deletions) which can independently deactivate the target β -fructofuranosidase gene.

This insertion vector is introduced in the cell to induce homologous recombination with the target β -fructofuranosidase gene on the chromosome between the two mutations. As a result, the chromosome now has two copies of the target β -fructofuranosidase gene, each having one mutation. The target β -fructofuranosidase gene is thus deactivated.

When using a substitution vector, a vector bearing the target β -fructofuranosidase gene which has been split by introducing a selectable marker gene is prepared.

The substitution vector is introduced in the cell to induce homologous recombination at two locations, with the selection marker in-between, in the region derived from the β -fructofuranosidase gene. As a result, the target β -fructofuranosidase gene on the chromosome is replaced with the gene containing the selectable marker gene and, thus, deactivated.

The two-step gene targeting is achieved either by direct substitution or hit-and-run substitution.

The first step of direct substitution is the same as the procedure using a substitution vector in one-step gene targeting. In the second step, a vector which bears a deactivated β -fructofuranosidase gene containing at least one mutation (preferably a deletion) which can independently deactivate the target β -fructofuranosidase gene is prepared. This vector is then introduced in the cell to induce homologous recombination at two locations, with the mutation in-between, in the target β -fructofuranosidase gene on the chromosome, which has been split by the selectable marker gene. As a result, the target β -fructofuranosidase gene on the chromosome is replaced with the deactivate target β -fructofuranosidase gene. These recombinant strains can be selected with the absence of the

marker gene as an index.

In the first step of hit-and-run substitution, a vector which bears a deactivated β -fructofuranosidase gene containing at least one mutation (preferably a deletion) which can independently deactivate the target β -fructofuranosidase gene and a selectable marker gene is prepared. This vector is then introduced in the cell to induce homologous recombination with the β -fructofuranosidase gene on the chromosome in the target β -fructofuranosidase gene on the upstream of the mutation. As a result, the vector bearing the selectable marker gene is now positioned between two copies of target β -fructofuranosidase gene on the chromosome — one with a mutation and one without. Next, the vector between the two copies of target β -fructofuranosidase gene is looped out, and allowed to homologously recombine again on the downstream of the mutation. As a result, the vector bearing the selectable marker gene and one copy of target β -fructofuranosidase gene is removed, leaving the target β -fructofuranosidase gene on the chromosome with a mutation. These recombinant strains can be selected with the absence of the marker gene as in index. It should be noted that the same effect is obviously achievable by inducing homologous recombination first on the downstream of the mutation, then on its upstream.

In the above procedures, any selectable marker gene may be used provided that a transformant is selectable. However, strains missing the selectable marker should be selected in the course of two-step gene targeting, it is preferable to use a selectable marker gene which allows these strains to be positively selected, such as nitrate reductase gene (*niaD*), orotidine-5'-phosphate decarboxylase gene (*pyrG*), or ATP sulfurylase gene (*sC*).

Examples of mold fungus according to the third aspect of the present invention include *Aspergillus niger* NIA1602 (FERM BP-5853).

Process for producing a recombinant β -fructofuranosidase using the mold fungus having no β -fructofuranosidase according to the third aspect of the present invention as a host

The mold fungus according to the present invention may preferably be used for producing recombinant β -fructofuranosidase.

More specifically, a DNA fragment encoding β -fructofuranosidase is introduced in the mold fungus according to the present invention in the form of a DNA molecule which is replicatable in the host cell according to the present invention and can express the gene, particularly an expression vector, in order to transform the mold fungus. The transformant has then the ability to produce the recombinant β -fructofuranosidase and no other β -fructofuranosidase enzymes.

This procedure, where a preferred form of the DNA molecule is a plasmid, may be carried out according to the standard techniques of genetic engineering.

According to a preferred embodiment of the present invention, examples of DNA fragments encoding β -fructofuranosidase include the DNA encoding β -fructofuranosidase according to the first aspect of the present invention as described earlier, the DNA encoding a novel β -fructofuranosidase which has been isolated in the process according to the second aspect of the present invention, and the DNA encoding a β -fructofuranosidase variant according to the fourth aspect of the present invention as described later.

Examples of systems for expressing β -fructofuranosidase using the mold fungus according to the third aspect as a host include the expressing system which has been described in the first aspect of the present invention.

More specifically, it is preferable that the plasmid to be used bear a selectable marker gene for the transformant, such as a drug-resistance marker gene or marker gene complementing an auxotrophic mutation. Examples of preferred marker genes include hygromycin-resistance gene (hph), bialaphos-resistance gene (Bar), nitrate reductase gene (niaD), orotidine-5'-phosphate decarboxylase gene (pyrG), and ATP-sulfurylase gene (sC).

It is also preferable that the DNA molecule for use as an expression vector contain nucleotide sequences necessary for the expression of the β -fructofuranosidase gene, including transcription and translation control signals, such as a promoter, a transcription initiation signal, a translation termination signal, and a transcription termination signal. Examples of preferred promoters include, in addition to the promoter on the inserted fragment which is able to

function in the host according to the present invention, promoters such as those of α -amylase gene (amy), glucoamylase gene (gla), β -fructofuranosidase gene, glyceraldehyde-3-phosphatase dehydrogenase gene (gpd), and phosphoglycerate kinase gene (pgk).

5 It is also advantageous to use a secretion vector as the expression vector to allow it to extracellularly secrete the produced recombinant β -fructofuranosidase.

In the system for producing β -fructofuranosidase using a mold fungus according to the third aspect of the present invention, the transformed mold fungus according to the present invention is first
10 cultivated under suitable conditions. The culture is treated by a known procedure such as centrifugation to obtain the supernatant or cell bodies. Cell bodies should be further suspended in a suitable buffer solution, then homogenized by freeze-and-thaw, ultrasonic
15 treatment, or mortar, followed by centrifugation or filtration to separate a cell body extract containing the novel recombinant β -fructofuranosidase.

β -Fructofuranosidase variant according to the fourth aspect of the present invention

20 The β -fructofuranosidase variant according to the fourth aspect of the present invention is obtained by the mutation of the original β -fructofuranosidase. In the present invention, the mutation comprises an insertion, substitution or deletion of one or more amino acids in, or an addition to either or both of the terminals
25 of, the amino acid sequence of the original β -fructofuranosidase, while the composition of the fructooligosaccharide mixture produced from sucrose as a result of fructosyltransfer reaction by the β -fructofuranosidase variant differs from the composition of the fructooligosaccharide mixture produced by the original
30 β -fructofuranosidase.

Although the source of the original β -fructofuranosidase is not limited in any way in the present invention provided that the β -fructofuranosidase has fructosyltransferase activity, it is preferable to use β -fructofuranosidase derived from Eumycetes, particularly
35 *Aspergillus*, *Penicillium*, *Scopulariopsis*, *Fusarium* or *Aureobasidium*. The most preferable β -fructofuranosidase is one derived from *Aspergillus*, particularly the β -fructofuranosidase consisting of the

amino acid sequence of SEQ ID No. 1 as shown in the sequence listing according to the first aspect of the present invention or a homologue thereof. The original β -fructofuranosidase may also be the β -fructofuranosidase which is obtained by the aforementioned
5 isolating process according to the second aspect of the present invention or a homologue thereof.

According to a preferred embodiment of the present invention, if the original β -fructofuranosidase consists of the amino acid sequence of SEQ ID No. 1, one such example is a variant in which one
10 or more amino acids selected from the group consisting of amino acid residues at positions 170, 300, 313 and 386 in the amino acid sequence are substituted by other amino acid residues.

According to a preferred embodiment of the present invention, preferred examples include variants in which:

15 the amino acid residue at position 170 is substituted by an aromatic amino acid selected from the group consisting of tryptophan, phenylalanine and tyrosine, most preferably tryptophan;

the amino acid residue at position 300 is substituted by an amino acid selected from the group consisting of tryptophan, valine,
20 glutamic acid and aspartic acid;

the amino acid residue at position 313 is substituted by a basic amino acid selected from the group consisting of lysine, arginine and histidine, most preferably lysine or arginine; and

25 the amino acid residue at position 386 is substituted by a basic amino acid selected from the group consisting of lysine, arginine and histidine, most preferably lysine. These variants are advantageous in that they can produce 1-kestose selectively and efficiently from sucrose.

The variants according to a more preferred embodiment of the present invention are those in which amino acid residues at positions 170, 300 and 313 are substituted by tryptophan, tryptophan and lysine, respectively, or by tryptophan, valine and lysine, respectively. These variants are advantageous in that they can produce 1-kestose more selectively and efficiently from sucrose.

35 If the original β -fructofuranosidase is a homologue of the amino acid sequence of SEQ ID No. 1, one such example is a variant in which one or more amino acid residues equivalent to the amino

acid residues at positions 170, 300, 313 and 386 in the amino acid sequence of SEQ ID No.1 are substituted by other amino acids. The amino acids to be substituted in a homologue of the original β -fructofuranosidase consisting of the amino acid sequence of SEQ ID No. 1 are easily selected by comparing amino acid sequences by a known algorithm. If, however, comparison of amino acid sequences by a known algorithm is difficult, the amino acids to be substituted can be easily determined by comparing the stereochemical structures of the enzymes.

Preparation of a variant β -fructofuranosidase according to the fourth aspect of the present invention

The variant β -fructofuranosidase according to the fourth aspect of the present invention may be prepared by procedures such as genetic engineering or polypeptide synthesis.

When employing genetic engineering, the DNA encoding the original β -fructofuranosidase is first obtained. Next, mutation is induced at specific sites on the DNA to substitute their encoded amino acids. Then, an expression vector containing the mutant DNA is introduced in a host cell to transform it. The transformant cell is cultivated to prepare the desired β -fructofuranosidase variant.

Several methods are known to those skilled in the art for inducing mutation at specific sites on a gene, such as the gapped duplex method (Methods in Enzymology, 154, 350 (1987)) and the Kunkel method (Methods in Enzymology, 154, 367 (1987)). These methods are applicable for the purpose of inducing mutation at specific sites on a DNA encoding β -fructofuranosidase. The nucleotide sequence of the mutant DNA may be identified by procedures such as the chemical degradation method devised by Maxam and Gilbert (Methods in Enzymology, 65, 499 (1980)) or the dideoxynucleotide chain termination method (Gene, 19, 269 (1982)). The amino acid sequence of the β -fructofuranosidase variant can be decoded from the identified nucleotide sequence.

Production of a β -fructofuranosidase variant according to the fourth aspect of the present invention

The β -fructofuranosidase variant according to the fourth aspect of the present invention may be produced in a host cell by introducing a DNA fragment encoding β -fructofuranosidase in the

host cell in the form of a DNA molecule which is replacatalbe in the host cell and can express the gene, particularly an expression vector, in order to transform the host cell.

- Therefore, the present invention provides a DNA molecule,
 5 particularly an expression vector, which comprises a gene encoding the β -fructofuranosidase variant according to the present invention. The DNA molecule is obtained by introducing a DNA fragment encoding the β -fructofuranosidase variant according to the present invention in a vector molecule. According to a preferred embodiment
 10 of the present invention, the vector is a plasmid.

The DNA molecule according to the present invention may be prepared by the standard technique of genetic engineering.

- The vector applicable in the present invention may be selected as appropriate, considering the type of the host cell used, from viruses,
 15 plasmids, cosmid vectors, etc. For example, a bacteriophage in the λ phage group or a plasmid in the pBR or pUC group may be used for E. coli host cells, a plasmid in the pUB group for Bacillus subtilis, and a vector in the YEp, YRp or YCp group for yeast.

- It is preferable that the plasmid contain a selectable marker to
 20 ease the selection of the transformant, such as a drug-resistance marker or marker gene complementing an auxotrophic mutation. Preferred examples of marker genes include ampicillin-resistance gene, kanamycin-resistance gene, and tetracycline-resistance gene for bacterium host cells; N-(5'-phosphoribosyl)-anthranilate
 25 isomerase gene (TRP1), orotidine-5'-phosphate decarboxylase (URA3), and β -isopropylmalate dehydrogenase gene (LEU2) for yeast; and hygromycin-resistance gene (hph), bialophos-resistance gene (Bar), and nitrate reductase gene (niaD) for mold.

- It is also preferable that the DNA molecule for use as an
 30 expression vector according to the present invention contain nucleotide sequences necessary for the expression of the β -fructofuranosidase gene, including transcription and translation control signals, such as a promoter, a transcription initiation signal, a ribosome binding site, a translation termination signal, and a
 35 transcription termination signal.

Examples of preferred promoters include, in addition to the promoter on the inserted fragment which is able to function in the

host, promoters such as those of lactose operon (lac), and tryptophan operon (trp) for *E. coli*; promoters such as those of alcohol dehydrogenase gene (ADH), acid phosphatase gene (PHO), galactose regulated gene (GAL), and glyceraldehyde-3-phosphate dehydrogenase gene (GPD) for yeast; and promoters such as those of α -amylase gene (amy), glucoamylase gene (gla), cellobiohydrolase gene (CBH1), and β -fructofuranosidase gene for mold.

If the host cell is *Bacillus subtilis*, yeast or mold, it is also advantageous to use a secretion vector to allow it to extracellularly secrete recombinant β -fructofuranosidase. Any host cell with an established host-vector system may be used, preferably yeast, mold, etc. The use of a host cell without sucrose metabolizing capability would be particularly preferred, as it does not have an enzyme which acts on sucrose except the expressed β -fructofuranosidase variant and, therefore, allows the resultant β -fructofuranosidase variant to be used for the production of fructooligosaccharides without purification. Thus, according to a preferred embodiment of the present invention, the mold fungus according to the third aspect of the present invention may be used as the host cell. A few *Trichoderma* strains and a type of yeast may be used as the host without sucrose metabolizing capability (Oda, Y. and Ouchi, K., Appl. Environ. Microbiol., 55, 1742-1747, 1989).

Production of fructooligosaccharides using the β -fructofuranosidase variant according to the fourth aspect of the present invention

The present invention further provides a process for producing fructooligosaccharides using the β -fructofuranosidase variant. The process for producing fructooligosaccharides is practiced by bringing the host cell which synthesizes the β -fructofuranosidase variant, or the β -fructofuranosidase variant itself into contact with sucrose.

In the process using the β -fructofuranosidase variant, fructooligosaccharides may be produced and purified under substantially the same conditions as in the process for producing fructooligosaccharides using the β -fructofuranosidase according to the first aspect of the present invention.

Examples

Example A

Example A1: Purification and partial sequencing of β -fructofuranosidase

An electrophoretically homogeneous sample of β -fructofuranosidase was obtained from the cell bodies of *Aspergillus niger* ACE-2-1 (ATCC20611) by purifying it according to the process described in Agric. Biol. Chem., 53, 667-673 (1989).

The purified enzyme was digested with lysyl endopeptidase (SKK Biochemicals Corp.). The resultant peptides were collected by HPLC (Waters) using a TSK gel ODS120T column (Tosoh Corp.), and sequenced using a protein sequencer (Shimadzu Corp.). As a result, four partial amino acid sequences were determined as shown in the sequence listing (SEQ ID Nos. 3 to 6).

The N-terminal of the enzyme protein before digested with lysyl endopeptidase was determined by using the protein sequencer as shown in the sequence listing (SEQ ID No. 7).

Example A2: Purification of partial DNA fragment of β -fructofuranosidase gene by PCR

Aspergillus niger ACE-2-1 (ATCC20611) was cultivated in a YPD medium (1% yeast extract, 2% polypepton and 2% glucose), then collected and freeze-dried. The homogenate was mixed with 8 ml of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA), then with 4 ml of TE buffer solution containing 10% SDS, and maintained at 60 °C for 30 minutes. Next, the solution was intensely shaken with a 12 ml mixture of phenol, chloroform and isoamyl alcohol (25:24:1), followed by centrifugation. The aqueous layer was transferred to another container, and mixed with 1 ml of 5M potassium acetate solution. After stored in an iced water bath for at least 1 hour, the solution was centrifuged. The aqueous layer was transferred to another container, and mixed with 2.5-fold volume of ethanol to sediment. The precipitate was dried and dissolved in 5 ml of TE buffer solution. After 5 μ l of 10 mg/ml RNase A (Sigma Chemical Co.) solution was added, the mixture was maintained at 37 °C for 1 hour. Then, 50 μ l of 20 mg/ml proteinase K (Wako Pure Chemical Industries, Ltd.) solution was added, and the mixture was maintained at 37 °C for 1 hour. Next, 3 ml of PEG solution (20% polyethylene glycol 6000 and 2.5 M sodium chloride) was added to sediment the DNA. The precipitate was dissolved in 500 μ l of TE buffer solution,

and extracted twice with a mixture of phenol, chloroform and isoamyl alcohol, then allowed to sediment in ethanol. This precipitate was washed in 70% ethanol, dried, then dissolved in an adequate amount of TE buffer solution (chromosomal DNA sample).

- 5 PCR was performed using Perkin Elmer Cetus DNA Thermal
Cycler as follows: The chromosomal DNA, 0.5 μ l (equivalent to 1 μ
g), which had been prepared above, was mixed with 10 μ l of buffer
solution [500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$ and
1% Triton X-100], 8 μ l of 2.5 mM dNTP solution, 1 μ l each of 1 mM
10 positive-chain DNA primer of SEQ ID No. 8 as shown in the sequence
listing (primer #1) and negative-chain DNA primer of SEQ ID No. 9 as
shown in the sequence listing (primer #2), 0.5 μ l Taq DNA
polymerase (Wako Pure Chemical Industries, Ltd.), and 79 μ l of
sterilized water, to a total volume of 100 μ l. After pretreatment at
15 94 °C for 5 minutes, the sample was incubated at 94 °C for 1 minute
(degeneration step), at 54 °C for 2 minutes (annealing step), and at 72 °
C for 3 minutes (extending step), for a total of 25 reaction cycles. The
last cycle was followed by incubation at 72 °C for 7 minutes. The
sample was then extracted with a mixture of phenol, chloroform and
20 isoamyl alcohol, and allowed to sediment in ethanol. The precipitate
was dissolved in 20 μ l of TE buffer solution and electrophoresed
through agarose gel. The specifically amplified band at about 800 bp
was cut out using the standard technique. The recovered DNA
fragment was allowed to sediment in ethanol.
- 25 After the DNA precipitate was dissolved in 8 μ l of sterilized
water, its terminals were blunted by using DNA Blunting Kit (Takara
Shuzo Co., Ltd.). Then, after the 5' terminal was phosphorylated
using T4 DNA kinase (Nippon Gene), the sequence was cloned to the
SmaI site of pUC119. The fragment inserted in the plasmid was
30 sequenced using a fluorescence sequencer, ALFred DNA Sequencer
(Pharmacia), as shown in the sequence listing (SEQ ID No. 10). The
total length of the PCR fragment was 788 bp. The first 14 amino
acids on the N terminal of the amino acid sequence encoded by this
DNA fragment corresponded to amino acids No. 7 to 20 of SEQ ID No.
35 3 as shown in the sequence listing, while amino acids No. 176 to 195
on the N terminal corresponded to amino acids No. 1 to 20 of SEQ ID
No. 4 as shown in the sequence listing. Further, the first 10 amino

acids on the C terminal of the same sequence corresponded to amino acids No. 1 to 10 of SEQ ID No. 5 as shown in the sequence listing. Thus, the amino acid sequence was identical to that determined from the purified β -fructofuranosidase.

5 Example A3: Screening of clone containing complete DNA fragment encoding β -fructofuranosidase

 About 10 μ g of chromosome DNA sample which had been prepared in Example A2 above was digested with EcoRI, followed by agarose gel electrophoresis, then blotted on a Hybond-N+ membrane
10 (Amersham International) according to the procedure described in Molecular Cloning (Cold Spring Harbour, 1982)

 This membrane was subjected to Southern analysis using ECL Direct DNA/RNA Labelling & Detection System (Amersham International), with the 788 bp PCR fragment prepared in Example A2
15 above used as a probe. As a result, a DNA fragment of about 15 kbp hybridized with the probe.

 In the next step, about 20 μ g of chromosomal DNA sample above was digested with EcoRI, followed by agarose gel electrophoresis. DNA fragments at about 15 kbp were separated and
20 recovered according to the procedure described in Molecular Cloning (Ibid.).

 The recovered DNA fragments of about 15 kbp (about 0.5 μ g) were ligated with 1 μ g of λ DASH II, which had been digested with both of HindIII and EcoRI, and packaged using an in vitro packaging kit, GIGAPACK II Gold (Stratagene L.L.C.), then introduced in E. coli
25 XLI-Blue MRA (P2), to prepare a library.

 As a result of plaque hybridization using ECL Direct DNA/RNA Labelling & Detection System (Amersham International) with the 788 bp PCR fragment above used as a probe, 25 clones turned out positive
30 in 15,000 plaques. Three of the positive clones were purified by a second screening to prepare phage DNA, which was then analyzed using restriction enzymes. The result showed that all the clones had an identical EcoRI fragment of about 15 kbp.

 This EcoRI fragment of about 15 kbp was subdivided into a
35 smaller fragment to select the desired DNA region using restriction enzymes, then subcloned to plasmid vector pUC118 or pUC119. The plasmid DNA was obtained from the subclone according to the

standard procedure and sequenced as in Example A2 using a fluorescence sequencer, ALFred DNA Sequencer (Pharmacia), as shown in the sequence listing (SEQ ID No. 2).

Example A4: Expression of β -fructofuranosidase gene by

5 *Trichoderma viride*

An about 5.5 kbp HindIII-XhoI fragment containing a gene encoding β -fructofuranosidase was prepared from the phage DNA obtained in Example A3. The fragment was ligated with the HindIII-SalI site of plasmid vector pUC119 (plasmid pAW20).

- 10 Further, plasmid pDH25 (D. Cullen et al., (1987) Gene, 57, 21-26) was partially digested with EcoRI and ligated with XbaI linker, and digested again with XbaI. Then, a 3 kbp XbaI fragment which consisted of the promoter and terminator of the trpC gene derived from *Aspergillus nidulans* and hygromycin B phosphotransferase
- 15 gene derived from *E. coli* was prepared as a hygromycin-resistance gene cassette. The fragment was inserted into the XbaI site of plasmid pAW20 (plasmid pAW20-Hyg in Figure 1).

- 20 *Trichoderma viride* was cultivated in a seed medium (3% glucose, 0.1% polypepton, 1% yeast extract, 0.14% ammonium sulfate, 0.2% potassium dihydrogenphosphate and 0.03% magnesium sulfate) at 28 °C for 20 hours. The resultant mycelium was collected by centrifugation at 3000 rpm for 10 minutes and washed twice in 0.5 M sucrose solution.

- 25 The mycelium was suspended in 0.5 M sucrose solution containing 5 mg/ml Cellulase-Onozuka R-10 (SKK Biochemicals Corp.) and 5 mg/ml of Novozym 234 (Novo Nordisk), and gently shaken at 30 °C for 1 hour to form protoplasts. After the cell body residue was filtered out, the suspension was centrifuged at 2500 rpm for 10 minutes. The collected protoplasts were washed twice in
- 30 SUTC buffer solution (0.5 M sucrose, 10 mM Tris-HCl (pH 7.5) and 10 mM calcium chloride) and suspended in the buffer solution to a final concentration of 10^7 /ml.

- 35 The protoplast suspension, 100 μ l, was mixed with 10 μ l of DNA solution, which had been dissolved in TE buffer solution so that the concentration of plasmid pAW20-Hyg would be 1 mg/ml, and iced for 5 minutes. Then, it was mixed with 400 μ l of PEG solution (60% polyethylene glycol 4000, 10 mM Tris-HCl (pH 7.5) and 10 mM

calcium chloride), and iced for an additional 20 minutes. Next, the protoplasts were washed in SUTC buffer solution, and laid on a potato dextrose agar medium (Difco) containing 100 μ g/ml hygromycin B and 0.5 M sucrose, together with a potato dextrose soft agar medium containing 0.5 M sucrose, and incubated at 28 °C for 5 days. The appeared colonies were selected as transformants.

After the transformant and the original strain were cultivated in the seed medium at 28 °C for 4 days, the β -fructofuranosidase activity of the culture supernatant was measured according to the method described in Agric. Biol. Chem., 53, 667-673 (1989). As a result, the original strain turned out negative for the activity, while the transformant exhibited 1×10^2 units/ml of activity.

Example B

Example B1: Southern analysis of chromosomal DNA from β -fructofuranosidase-producing fungi

(1) Preparation of DNA fragment for use as probe

A DNA fragment for use as a probe was prepared by PCR, with plasmid pAW20-Hyg containing the nucleotide sequence of SEQ ID No. 2 as shown in the sequence listing as template DNA. PCR was performed with Perkin Elmer Cetus DNA Thermal Cycler as follows: The plasmid DNA (pAW20-Hyg), 0.5 μ l (equivalent to 0.1 μ g), which had been prepared above, was mixed with 10 μ l of reaction buffer solution [500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM MgCl₂ and 1% Triton X-100], 8 μ l of 2.5 mM dNTP solution, 2 μ l each of 0.01 mM positive-chain DNA primer of SEQ ID No. 15 as shown in the sequence listing (primer #1) and negative-chain DNA primer of SEQ ID No. 16 as shown in the sequence listing (primer #2), 0.5 μ l Taq DNA polymerase (Wako Pure Chemical Industries, Ltd.), and 77 μ l of sterilized water, to a total volume of 100 μ l. After pretreatment at 94 °C for 5 minutes, the sample was incubated at 94 °C for 1 minute (degeneration step), at 54 °C for 2 minutes (annealing step), and at 72 °C for 3 minutes (extending step), for a total of 25 reaction cycles. The last cycle was followed by incubation at 72 °C for 7 minutes. The sample was then extracted with a mixture of phenol, chloroform and isoamyl alcohol, and allowed to sediment in ethanol. The precipitate was dissolved in 20 μ l of TE buffer solution and electrophoresed through agarose gel. The specifically amplified band at about 2 kbp

was cut out using the standard technique. The recovered DNA fragment was allowed to sediment in ethanol. The DNA precipitate was dissolved in sterilized water to a concentration of $0.1 \mu\text{g}/\mu\text{l}$ to obtain a sample solution.

5 (2) Preparation and Southern Analysis of chromosomal DNA from β -fructofuranosidase-producing fungi

Mold fungus strains having the capability to produce β -fructofuranosidase: *Aspergillus japonicus* IFO4408, *Aspergillus aculeatus* IFO31348, *Penicillium roqueforti* IAM7254, *Scopulariopsis brevicaulis* IFO4843, IFO5828, IFO5841, IFO6588, IFO31688 and IFO31915, *Scopulariopsis brevicaulis* var. *glabra* IFO7239, and *Scopulariopsis roseola* IFO7564, were cultivated in a YPD liquid medium (1% yeast extract, 2% polypepton and 2% glucose) at 28°C for 2 days. From the resultant cell bodies, the chromosomal DNA
15 was prepared according to the procedure described in Example A2. About $10 \mu\text{g}$ each of the chromosomal DNA samples was digested with EcoRI, followed by agarose gel electrophoresis, then blotted on a Hybond-N+ membrane (Amersham International) according to the procedure described in Molecular Cloning (Ibid.).

20 This membrane was subjected to the Southern analysis using ECL Direct DNA/RNA Labelling & Detection System (Amersham International), with the about 2 kbp DNA fragment prepared in (1) above used as a probe. The result showed that there was a DNA fragment which hybridized with the probe at about 20 kbp in
25 *Aspergillus japonicus* IFO4408, at about 13 kbp in *Aspergillus aculeatus* IFO31348, at about 4 kbp in *Penicillium roqueforti* IAM7254, at about 10 kbp in *Scopulariopsis brevicaulis* IFO4843, IFO5828, IFO5841, IFO6588, IFO31688 and IFO31915s, at about 2.7 kbp in *Scopulariopsis brevicaulis* var. *glabra* IFO7239, and at about
30 10 kbp in *Scopulariopsis roseola* IFO7564. This result indicated that a β -fructofuranosidase gene can be isolated from a β -fructofuranosidase-producing fungus by making use of its homology to the nucleotide sequence of SEQ ID No. 2 as shown in the sequence listing.

35 Example B2: Isolation of β -fructofuranosidase gene from *Penicillium roqueforti* IAM7254

About $20 \mu\text{g}$ of chromosomal DNA sample derived from

Penicillium roqueforti IAM7254 was digested with EcoRI, followed by agarose gel electrophoresis. DNA fragments at about 4 kbp were separated and recovered according to the procedure described in Molecular Cloning (Ibid.).

- 5 The recovered DNA fragments of about 4 kbp (about 0.5 μ g) were ligated with 1 μ g of λ gt 10 vector, which had been digested with EcoRI and treated with phosphatase, and packaged using an in vitro packaging kit, GIGAPACK II Gold (Stratagene L.L.C.), then introduced in the E. coli NM514 to prepare a library. As a result of
- 10 plaque hybridization using ECL Direct DNA/RNA^o Labelling & Detection System (Amersham International) with the about 2 kbp DNA fragment prepared in Example B1 used as a probe, four clones turned out positive in about 25,000 plaques. The positive clones were purified by a second screening to prepare phage DNA, which was
- 15 then analyzed using restriction enzymes. The result showed that all the clones had an identical EcoRI fragment of about 4 kbp.

- The about 4 kbp EcoRI fragment was subdivided into a smaller fragment to select the desired DNA region using restriction enzymes, then subcloned to plasmid vector pUC118 or pUC119. The plasmid
- 20 DNA was obtained from the subclone according to the standard procedure and sequenced using a fluorescence sequencer, ALFred DNA Sequencer (Pharmacia) as shown in the sequence listing (SEQ ID No. 12). The encoded amino acid sequence was as shown in the sequence listing (SEQ ID No. 11).

- 25 **Example B3: Isolation of β -fructofuranosidase gene from Scopulariopsis brevicaulis IFO4843**

- About 20 μ g of chromosomal DNA sample derived from Scopulariopsis brevicaulis IFO4843 was digested with EcoRI, followed by agarose gel electrophoresis. DNA fragments at about 10 kbp were
- 30 separated and recovered according to the procedure described in Molecular Cloning (Ibid.).

- The recovered DNA fragments of about 10 kbp (about 0.5 μ g) were ligated with 1 μ g of λ DASH II vector, which had been digested with both of HindIII and EcoRI, and packaged using an in vitro
- 35 packaging kit, GIGAPACK II Gold (Stratagene L.L.C.), then introduced in E. coli XLI-Blue MRA (P2), to prepare a library.

As a result of plaque hybridization using ECL Direct DNA/RNA

Labelling & Detection System (Amersham International) with the about 2 kbp DNA fragment prepared in Example B1 used as a probe, three clones turned out positive in about 15,000 plaques. The positive clones were purified by a second screening to prepare phage DNA, which was then analyzed using restriction enzymes. The result showed that all the clones had an identical EcoRI fragment of about 10 kbp.

The about 10 kbp EcoRI fragment was subdivided into a smaller fragment to select the desired DNA region using restriction enzymes, then subcloned to plasmid vector pUC118 or pUC119. The plasmid DNA was obtained from the subclone according to the standard procedure and sequenced using a fluorescence sequencer, ALFred DNA Sequencer (Pharmacia) as shown in the sequence listing (SEQ ID No. 14). The encoded amino acid sequence was as shown in the sequence listing (SEQ ID No. 13).

Example B4: Expression of β -fructofuranosidase gene derived from *Penicillium roqueforti* IAM7254 in *Trichoderma viride*

An about 4 kbp EcoRI fragment containing a gene encoding β -fructofuranosidase was prepared from the phage DNA obtained in Example B2. The fragment was inserted into the EcoRI site of plasmid vector pUC118 (plasmid pPRS01).

Further, plasmid pDH25 (D. Cullen et al., (1987) Gene, 57, 21-26) was partially digested with EcoRI and ligated with XbaI linker, and digested again with XbaI. Then, a 3 kbp XbaI fragment which consisted of the promoter and terminator of the trpC gene derived from *Aspergillus nidulans* and hygromycin B phosphotransferase gene derived from *E. coli* was prepared as a hygromycin-resistance gene cassette. The fragment was inserted into the XbaI site of plasmid pPRS01 (plasmid pPRS01-Hyg in Figure 2).

Trichoderma viride was cultivated in a seed medium (3% glucose, 0.1% polypepton, 1% yeast extract, 0.14% ammonium sulfate, 0.2% potassium dihydrogenphosphate and 0.03% magnesium sulfate) at 28°C for 20 hours. The resultant mycelium was collected by centrifugation at 3000 rpm for 10 minutes and washed twice in 0.5 M sucrose solution.

The mycelium was suspended in 0.5 M sucrose solution containing 5 mg/ml of Cellulase-Onozuka R-10 (Yakult) and 5

mg/ml of Novozym 234 (Novo Nordisk), and gently shaken at 30 ° C for 1 hour to form protoplasts. After the cell body residue was filtered out, the suspensions were centrifuged at 2500 rpm for 10 minutes. The collected protoplasts were washed twice in SUTC buffer solution (0.5 M sucrose, 10 mM Tris-HCl (pH 7.5) and 10 mM calcium chloride) and suspended in the buffer solution to a final concentration of 10^7 /ml.

The protoplast suspension, 100 μ l, was mixed with 10 μ l of DNA solution, which had been dissolved in TE buffer solution so that the concentration of plasmid pPRS01-Hyg would be 1 mg/ml, and iced for 5 minutes. Then, it was mixed with 400 μ l of PEG solution (60% polyethylene glycol 4000, 10 mM Tris-HCl (pH 7.5) and 10 mM calcium chloride), and iced for an additional 20 minutes. Next, the protoplasts were washed in SUTC buffer solution, and laid on a potato dextrose agar medium (Difco) containing 100 μ g/ml hygromycin B and 0.5 M sucrose, together with a potato dextrose soft agar medium containing 0.5 M sucrose, and incubated at 28 ° C for 5 days. The appeared colonies were selected as transformants.

After the transformant and the original strain were cultivated in the seed medium at 28 ° C for 4 days, the β -fructofuranosidase activity of the culture supernatant was measured by allowing the enzyme to act on 10 wt% sucrose solution, pH 5.5, at 40 ° C. The activity was expressed in units, i.e., the quantity of free glucose (μ mol) released in 1 minute. The original strain turned out negative for the activity, while the transformant exhibited about 0.04 units/ml of activity.

The obtained β -fructofuranosidase was allowed to act on sucrose for 23 hours at 40 ° C in a sucrose solution at a concentration of 60 wt%, pH 7.0, containing 4.2 units of enzyme per 1 g of sucrose. After the reaction, the sugar composition in the solution was 1.6% fructose, 16.2% glucose, 42.3% sucrose, 37.3% GF2 and 2.1% GF3.

Example C

Example C1: Preparation of niaD transformant from *Aspergillus niger* ACE-2-1

Spores of *Aspergillus niger* ACE-2-1 (ATCC20611) were applied to a minimal agar medium (0.2% sodium glutamate, 0.1% dipotassium hydrogenphosphate, 0.05% magnesium sulfate, 0.05%

potassium chloride, 0.001% iron sulfate, 3% sucrose and 0.5% agar, pH 5.5) containing 6% chlorates, and maintained at 30 ° C. After incubation for about 5 days, strains which formed colonies (chlorate-resistant mutants) were selected and planted in a minimal medium which contained glutamates, nitrates or nitrites as the only nitrogen source for the examination of their requirement for nitrogen source. The result showed that some of the chlorate-resistant mutants (niaD mutant candidates) were able to grow in the minimal medium containing glutamates or nitrites as the only nitrogen source, but not in the one containing nitrates.

Three strains of the niaD mutant candidates were analyzed for the activity of nitrate reductase, which was supposed to be produced by niaD gene, in the cell body. The three strains were cultivated in a liquid medium (0.2% sodium glutamate, 0.1% dipotassium hydrogenphosphate, 0.05% magnesium sulfate, 0.05% potassium chloride, 0.001% iron sulfate and 3% sucrose 3 g) at 30 ° C for 60 hours while shaking. The resultant wet cell bodies, 0.2g, were suspended in 2 ml of 50 mM sodium phosphate buffer (pH 7.5), homogenized, and ultrasonically crushed, then centrifuged to remove the insoluble fraction. The supernatant, 50 μ l, was mixed with 1000 μ l of distilled water, 750 μ l of 0.2 M sodium phosphate solution (pH 7.5), 100 μ l of 0.04 mg/ml FAD, 100 μ l of 2 mg/ml NADPH and 1000 μ l of 22.5 mg/ml sodium nitrate, and allowed to react at 37 ° C. After reaction was over, the sample solution was colored by the addition of 500 μ l of 1% sulfanilamide (dissolved in 3 N hydrochloric acid) and 500 μ l of 0.02% N-1-naphthylethylenediamine, and measured for A540 for the determination of the nitrate reductase activity. However, these three strains did not exhibit nitrate reductase activity. Therefore, it was concluded that the three strains were niaD mutants, one of which, named NIA5292 strain, was used as a sample in the subsequent experiments.

Example C2: Preparation of niaD gene from *Aspergillus niger* NRRL4337

(1) Preparation of probe

Aspergillus niger NRRL4337 was cultivated in a YPD liquid medium (1% yeast extract, 2% polypepton and 2% glucose). Further,

synthetic DNA primers as shown in the sequence listing (SEQ ID Nos. 17 and 18) were prepared by referring to the nucleotide sequence of *niaD* gene derived from *Aspergillus niger* (Unkdes, S. E., et al., Gene 111, 149-155 (1992)). The chromosomal DNA which had been prepared from the aforementioned cell bodies according to the procedure described in Example A2 was used as a template DNA for PCR reaction. The reaction took place in 100 μ l of sample solution containing 0.5 μ g of chromosomal DNA, 100 pmol each of primers and 2.5U of Taq DNA polymerase (Nippon Gene) at 94 °C for 1 minute, at 50 °C for 2 minutes, and at 72 °C for 2 minutes, for a total of 25 cycles. As a result, an about 800 bp DNA fragment was amplified specifically. Then, the nucleotide sequence of this DNA fragment was analyzed and proved to be identical to the reported nucleotide sequence of the *niaD* gene of *Aspergillus niger*, showing that the DNA fragment was derived from the *niaD* gene. This about 800 bp DNA fragment was used as a probe in the subsequent experiments.

(2) Southern analysis of chromosomal DNA from *Aspergillus niger*

The chromosomal DNA of *Aspergillus niger* NRRL4337 was digested completely with HindIII, EcoRI and BamHI, followed by electrophoretic fractionation on agarose gel, then blotted on a nylon membrane (Hybond-N+, Amersham International) according to the procedure described in Molecular Cloning (Cold Spring Harbour, 1982). This nylon membrane subjected to Southern analysis using ECL Direct DNA Labelling & Detection System (Amersham International) under the conditions specified in the supplied manual, with the aforementioned about 800 bp DNA fragment used as a probe. As a result, a DNA fragment of about 15 kbp digested with HindIII hybridized with the probe.

(3) Isolation of *niaD* gene

The chromosomal DNA of the *Aspergillus niger* NRRL4337 was digested completely with HindIII, followed by electrophoretic fractionation on agarose gel. DNA fragments at about 15 kbp were separated and recovered according to the standard procedure. The recovered DNA fragments were ligated with the HindIII site of λ DASH II, and packaged using GIGAPACK II Gold (Stratagene L.L.C.), then introduced in *E. coli*, to prepare a library.

As a result of plaque hybridization using ECL Direct DNA Labelling & Detection System (Amersham International) with the about 800 bp DNA fragment above used as a probe, positive clones were obtained. The positive clones were purified by a second screening.

- 5 Phage DNA prepared from the positive clones were tested positive for a HindIII inserted fragment of about 15 kbp. As a result of Southern Analysis for this inserted fragment, a smaller DNA fragment of about 6.5 kbp containing the *niaD* gene (*XbaI* fragment) was found. A restriction enzyme map was determined for this fragment. Then, the *XbaI* fragment was subdivided into smaller fragments using restriction enzymes, and subcloned to plasmid pUC118. Using the subcloned plasmids as templates, the fragments were sequenced to determine the location of the *niaD* gene in the isolated DNA fragment (Figure 3).

15 Example C3: Construction of plasmid pAN203 for gene targeting

Plasmid pAN203 for gene targeting was constructed as follows (Figure 4):

- 20 An about 3 kbp *Sall* fragment including the initiation codon of the β -fructofuranosidase gene and its upstream region was prepared from the about 15 kbp *EcoRI* fragment containing a β -fructofuranosidase gene, which had been obtained in Example A3 above, and subcloned to plasmid PUC119 (plasmid pW20).
25 Single-stranded DNA was prepared from this plasmid, and site-specifically mutated using the synthetic DNA of SEQ ID No. 19 as shown in the sequence listing and Sculptor In Vitro Mutagenesis System (Amersham International), to create a *Bam*HI-digestible site immediately before the initiation codon of the β -fructofuranosidase gene (pW20B).
30

- Further, an about 1.5 kbp *PstI* fragment containing the termination codon of the β -fructofuranosidase gene and its downstream region was prepared from an about 15 kbp *EcoRI* fragment containing the β -fructofuranosidase gene, and subcloned to plasmid pUC119 (plasmid pBW20). single-stranded DNA was prepared from this plasmid, and site-specifically mutated using the synthetic DNA of SEQ ID No. 20 as shown in the sequence listing and
35

Sculptor In Vitro Mutagenesis System (Amersham International), to create a BamHI-digestible site immediately after the termination codon of the β -fructofuranosidase gene (pBW20B). An about 1.5 kbp PstI fragment was prepared from pBW20B and substituted for the about 1.5 kbp PstI fragment of pAW20, which had been prepared in Example A4 (plasmid pAW20B).

Next, plasmid pUC118 was digested with HindIII and, after its terminals were blunted with T4 DNA polymerase (Takara Shuzo Co., Ltd.), ligated with Sall linker. The DNA was digested with Sall and ligated again (plasmid pUC18PHd). Plasmid pUC18PHd was digested with Sall and EcoRI, and ligated with an about 2.5 kbp Sall-BamHI fragment prepared from pW20B and an about 3 kbp BamHI-EcoRI fragment prepared from pAW20B (plasmid pAN202). Further, an about 6.5 kbp XbaI fragment (Figure 3) containing the *niaD* gene was inserted into the XbaI site of pAN202 (plasmid pAN203).

Example C4: Transformation of *Aspergillus niger* NIA5292 with Plasmid pAN203

Aspergillus niger NIA5292 was cultivated in a liquid medium (2% soluble starch, 1% polypepton, 0.2% yeast extract, 0.5% sodium dihydrogenphosphate and 0.05% magnesium sulfate) at 28 °C for 24 hours with shaking. The cell bodies were collected with a glass filter, suspended in an enzyme solution (1 mg/ml β -glucuronidase (Sigma Chemical Co.), 5 mg/ml Novozym 234 (Novo Nordisk), 10 mM sodium phosphate (pH 5.8) and 0.8M potassium chloride), and maintained at 30 °C for 1.5 hours. After the cell debris was removed by a glass filter, and the resultant protoplasts were collected by centrifugation. The protoplasts were washed twice in STC buffer (10 mM Tris (pH 7.5), 10 mM calcium chloride and 1.2 M sorbitol), and suspended in STC buffer. Next, the protoplasts were mixed with plasmid pAN203 which had been digested with HindIII, and maintained still on ice for 20 minutes. After PEG solution (10 mM Tris (pH 7.5), 10 mM calcium chloride and 60% polyethylene glycol 6000) was added, the sample was maintained still on ice for another 20 minutes. The protoplasts were washed a few times in STC buffer, and suspended in Czapek's medium (0.2% sodium nitrate, 0.1% dipotassium hydrogenphosphate, 0.05% magnesium sulfate, 0.05% potassium

chloride, 0.001% ferric sulfate and 3% sucrose) containing 1.2 M sorbitol and 0.8% agar. It was then overlaid on Czapek's agar medium containing 1.2 M sorbitol and 1.5% agar, and incubated at 30 ° C. After incubation for about 5 days, strains which formed colonies (transformants) were selected and cultivated in a liquid medium. The chromosomal DNAs of the transformants were extracted and analyzed by the Southern method, in order to select transformant in which only one copy of plasmid pAN203 was inserted by homologous recombination in the upstream region of the host β -fructofuranosidase gene.

Next, the conidia of the transformant were applied to a minimal agar medium (0.2% sodium glutamate, 0.1% dipotassium hydrogenphosphate, 0.05% magnesium sulfate, 0.05% potassium chloride, 0.001% iron sulfate, 2% glucose, 6% potassium chlorate and 1.5% agar, pH 5.5) which contained 6% potassium chlorate and 2% glucose as the only carbon source, and incubated at 30 °C. About four days later, a number of chlorate-resistant *niaD*⁻ phenotype mutants emerged. About half of the chlorate-resistant mutants were tested negatively for β -fructofuranosidase activity, suggesting that the β -fructofuranosidase gene was missing together with the vector bearing the *niaD* gene as a result of a secondary homologous recombination in the downstream region of the β -fructofuranosidase gene on the host chromosome. The result of Southern Analysis for the chromosomal DNA extracted from the chlorate-resistant mutants (one of which was named NIA1602) confirmed that the β -fructofuranosidase gene and the vector bearing the *niaD* gene were missing in the chromosome.

Example C5: Production of β -fructofuranosidase derived from *Penicillium roqueforti* in *Aspergillus niger* NIA1602 Host

To express the β -fructofuranosidase gene derived from *Penicillium roqueforti*, plasmid pAN572 was constructed as follows (Figure 5): First, plasmid pUC18 was digested with HindIII and, after its terminals were blunted with T4 DNA polymerase (Takara Shuzo Co., Ltd.), ligated again. Then, the plasmid was digested with BamHI and, after its terminals were blunted by T4 DNA polymerase, ligated again (plasmid pUC18HBX). An about 2 kbp PstI fragment containing the promoter and terminator of the β -fructofuranosidase

gene prepared from plasmid pAN202 was inserted into the PstI site of plasmid pUC18HBX (plasmid pAN204).

Next, in order to make a smaller DNA fragment of the *niaD* gene and disrupt the BamHI-digestible site, the gene was site-specifically mutated using the synthetic DNA of SEQ ID Nos. 21 and 22 as shown in the sequence listing as primers and Sculptor In Vitro Mutagenesis System (Amersham International). As a result, the BamHI-digestible site was disrupted and an XbaI-digestible site was created on the downstream of the *niaD* gene, allowing the *niaD* gene to be prepared as an about 4.8 kbp XbaI fragment without a BamHI-digestible site. This 4.8 kbp XbaI fragment was inserted into the XbaI site of plasmid pAN204 (plasmid pAN205).

Further, the translated region of the β -fructofuranosidase gene derived from *Penicillium roqueforti* was site-specifically mutated to disrupt the BamHI site without changing the encoded amino acid sequence (pPRS02). Mutation took place on Sculptor In Vitro Mutagenesis System (Amersham International), with the single-stranded DNA which had been prepared in Example B4 from plasmid pPRS01 containing the gene used as a template, and the synthetic DNA of SEQ ID No. 23 as shown in the sequence listing used as a primer. Then, an about 1.8 kbp BamHI fragment was prepared from the translated region of the β -fructofuranosidase gene by PCR using the synthetic DNA of SEQ ID No. 24 and 25 as shown in the sequence listing as primers and plasmid pPRS02 as template, and inserted into the BamHI site of plasmid pAN205 (plasmid pAN572).

Aspergillus niger NIA1602 was transformed according to the procedure described in Example C4 by using plasmid pAN572 which had been digested with HindIII to linearize. One of the transformants was cultivated in a liquid medium (5.0% sucrose, 0.7% malt extract, 1.0% polypepton, 0.5% carboxymethyl cellulose and 0.3% sodium chloride) at 28 °C for 3 days. After cultivation, the recovered cell bodies were ultrasonically homogenized, and measured for β -fructofuranosidase activity in units, i.e., the quantity of free glucose (μ mol) released in 1 minute in 10 wt% sucrose solution, pH 5.5, at 40 °C. The transformant exhibited 1×10^{-3} units/ml of activity.

Example D.

For ease of reference, a β -fructofuranosidase variant is hereinafter denoted by the following:

Original amino acid / position / Substitutional amino acid

According to this, for example, a variant in which tryptophan is substituted for phenylalanine at position 170 is expressed as "F170W."

A variant with more than one mutation is denoted by a series of mutation symbols separated by a '+', such as in:

F170W+G300V+H313K

where tryptophan, valine and lysine are substituted for phenylalanine, glycine and histidine at positions 170, 300 and 313, respectively.

Further, fructose, glucose and sucrose are hereinafter denoted by 'F', 'G', 'GF', respectively, while oligosaccharides in which one to three molecules of fructose are coupled with sucrose are denoted by 'GF2', 'GF3', and 'GF4', respectively.

Example D1: Construction and production of F170W variant

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

The translated region of the β -fructofuranosidase gene derived from *Aspergillus niger* ACE-2-1 (ATCC20611) was amplified by PCR using Perkin Elmer Cetus DNA Thermal Cycler, with plasmid pAW20-Hyg (see Example A4) containing the β -fructofuranosidase gene used as template DNA. The sample solution contained 0.5 μ l (equivalent to 0.1 μ g) of plasmid DNA (pAW20-Hyg), 10 μ l of reaction buffer solution [500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM MgCl₂ and 1% Triton X-100], 8 μ l of 2.5 mM dNTP solution, 2 μ l each of 0.01 mM positive-chain DNA primer of SEQ ID No. 26 as shown in the sequence listing (primer #1) and negative-chain DNA primer of SEQ ID No. 27 as shown in the sequence listing (primer #2), 0.5 μ l Taq DNA polymerase (Wako Pure Chemical Industries, Ltd.), and 77 μ l of sterilized water, with a total volume of 100 μ l. After pretreatment at 94 °C for 5 minutes, the sample was incubated at 94 °C for 1 minute (degeneration step), at 54 °C for 2 minutes (annealing step), and at 72 °C for 3 minutes (extending step), for a total of 25 reaction cycles. The last cycle was followed by incubation at 72 °C for 7 minutes. The sample was then extracted with a mixture of phenol,

chloroform and isoamyl alcohol, and allowed to sediment in ethanol. The precipitate was dissolved in 20 μ l of TE buffer solution and electrophoresed through agarose gel. The specifically amplified band at about 2 kbp was cut out using the standard technique. The recovered DNA fragment was digested with BamHI, then inserted into the BamHI site of plasmid pUC118 (Takara Shuzo Co., Ltd.) (plasmid pAN120 in Figure 6).

Plasmid pAN120 was introduced in the *E. coli* CJ236 strain to prepare single-stranded DNA according to the standard procedure. With the obtained DNA used as a template and the DNA primer of SEQ ID No. 28 as shown in the sequence listing as a primer, a site specific mutation was induced by using Muta-Gene In Vitro Mutagenesis Kit (Nihon Bio-Rad Laboratories) according to the instructions given in the supplied manual (plasmid pAN120 (F170W)).

The result of sequencing for the inserted fragment of pAN120 (F170W) confirmed that substitution occurred only in the target nucleotide and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that tryptophan was substituted for phenylalanine at position 170.

(2) Construction of expression vector pY2831 for use in yeast

Expression vector pY2831 for use in yeast was prepared from plasmid pYPR2831 (H. Horiuchi et al., *Agric. Biol. Chem.*, 54, 1771-1779, 1990). As shown in Figure 7, the plasmid was first digested with EcoRI and Sall and, after its terminals were blunted with T4DNA polymerase, ligated with BamHI linker (5'-CGGATCCG-3'), then digested again with BamHI and finally self-ligated (plasmid pY2831).

(3) Production of variant F170W by yeast

A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (F170W) by digesting it with BamHI, and inserted into the BamHI site of pY2831 (plasmid pYSUC (F170W) in Figure 8). A plasmid for expressing the wild type enzyme (plasmid pYSUC) was constructed in a similar manner from Plasmid pAN120.

These plasmids were introduced in the yeast *Saccharomyces cerevisiae* MS-161 (Suc⁻, ura3, trp1) by the lithium acetate method (Ito, H. et al., *J. Bacteriol.*, 153, 163-168, 1983) to prepare a

transformant. The transformant was cultivated overnight in an SD-Ura medium (0.67% yeast nitrogen base (Difco), 2% glucose and 50 μ g/ml uracil) at 30 °C. The culture was seeded in a production medium (0.67% yeast nitrogen base (Difco), 2% glucose, 2% casamino acids and 50 μ g/ml uracil) at a final concentration of 1% and cultivated at 30 °C for 2 days. The culture supernatant was measured for β -fructofuranosidase activity according to the procedure described in Agric. Biol. Chem., 53, 667-673 (1989). The activity was 12.7 units/ml in the wild type enzyme, and 10.1 units/ml in the F170W variant.

(4) Evaluation of variant F170W

The wild type enzyme and the variant F170W were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 °C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and the variant when 1-kestose (GF2) yield was at maximum were as follows:

	F	G	GF	GF2	GF3	GF4
Wild type	0.4	22.3	20.5	45.1	11.3	0.3
F170W	0.6	22.1	20.9	45.8	10.3	0.3

These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in F170W.

Example D2: Construction and production of variant G300W

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

A site specific mutation was induced in the same manner as in Example D1 except that the DNA primer of SEQ ID No. 29 as shown in the sequence listing was used to construct plasmid pAN120 (G300W).

The result of sequencing for the inserted fragment of pAN120 (G300W) confirmed that substitution occurred only in the target nucleotide and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that tryptophan was substituted for glycine at position 300.

(2) Production of variant G300W by yeast

A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (G300W) by digesting it with BamHI, and inserted into the BamHI site of pY2831 (plasmid pYSUC (G300W)).

- 5 Plasmid pYSUC (G300W) was introduced in the yeast *Saccharomyces cerevisiae* MS-161 in the same manner as in Example D1 to produce variant G300W. The culture supernatant exhibited a β -fructofuranosidase activity of 5.0 units/ml.

(3) Evaluation of variant G300W

- 10 The wild type enzyme and the variant G300W were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 °C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and the variant when 1-kestose (GF2) yield was at maximum were as follows:

	F	G	GF	GF2	GF3	GF4
Wild type	0.4	22.3	20.5	45.1	11.3	0.3
G300W	0.6	21.9	21.7	46.4	9.4	0.0

- 20 These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in G300W.

Example D3: Construction and production of variant H313K

- (1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

- 25 A site specific mutation was induced in the same manner as in Example D1 except that the DNA primer of SEQ ID No. 30 as shown in the sequence listing was used to construct plasmid pAN120 (H313K).

- 30 The result of sequencing for the inserted fragment of pAN120 (H313K) confirmed that substitution occurred only in the target nucleotide and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that lysine was substituted for histidine at position 313.

- 35 (2) Production of variant H313K by yeast

A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (H313K) by digesting it with BamHI,

and inserted into the BamHI site of pY2831 (plasmid pYSUC (H313K)).

- Plasmid pYSUC (H313K) was introduced in the yeast *Saccharomyces cerevisiae* MS-161 in the same manner as in Example D1 to produce variant H313K. The culture supernatant exhibited a β -fructofuranosidase activity of 5.0 units/ml.

- (3) Evaluation of variant H313K The wild type enzyme and the variant H313K were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 ° C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and the variant when 1-kestose (GF2) yield was at maximum were as follows:

	F	G	GF	GF2	GF3	GF4
15 Wild type	0.4	22.3	20.5	45.1	11.3	0.3
H313K	0.4	21.9	18.8	52.9	6.0	0.0

These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in H313K.

- Example D4: Construction and production of variant E386K

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

- A site specific mutation was induced in the same manner as in Example D1 except that the DNA primer of SEQ ID No. 31 as shown in the sequence listing was used to construct plasmid pAN120 (E386K).

- The result of sequencing for the inserted fragment of pAN120 (E386K) confirmed that substitution occurred only in the target nucleotide and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that lysine was substituted for glutamic acid at position 386.

(2) Production of variant E386K by yeast

- A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (E386K) by digesting it with BamHI, and inserted into the BamHI site of pY2831 (plasmid pYSUC (E386K)).

Plasmid pYSUC (E386K) was introduced in the yeast

Saccharomyces cerevisiae MS-161 in the same manner as in Example D1 to produce variant E386K. The culture supernatant exhibited a β -fructofuranosidase activity of 10.7 units/ml.

(3) Evaluation of variant E386K

- 5 The wild type enzyme and the variant E386K were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 °C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and the variant when 1-kestose (GF2) yield was at maximum were as follows:

10

	F	G	GF	GF2	GF3	GF4
Wild type	0.4	22.3	20.5	45.1	11.3	0.3
15 E386K	22.3	(F+G)	19.9	49.3	7.9	0.6

These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in E386K.

- 20 Example D5: Construction and production of variant F170W+G300W

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

- Site specific mutations were induced in the same manner as in Example D1 except that the DNA primers of SEQ ID Nos. 28 and 29 as shown in the sequence listing were used to construct plasmid pAN120 (F170W+G300W).

- 30 The result of sequencing for the inserted fragment of pAN120 (F170W+G300W) confirmed that substitution occurred only in the target nucleotides and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that tryptophan was substituted for phenylalanine at position 170 and glycine at position 300.

(2) Production of variant F170W+G300W by yeast

- 35 A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (F170W+G300W) by digesting it with BamHI, and inserted into the BamHI site of pY2831 (plasmid pYSUC (F170W+G300W)).

Plasmid pYSUC (F170W+G300W) was introduced in the yeast *Saccharomyces cerevisiae* MS-161 in the same manner as in Example D1 to produce variant F170W+G300W. The culture supernatant exhibited a β -fructofuranosidase activity of 2.3 units/ml.

5 (3) Evaluation of variant F170W+G300W

The wild type enzyme and the variant F170W+G300W were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 °C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and
10 the variant when 1-kestose (GF2) yield was at maximum were as follows:

	F	G	GF	GF2	GF3	GF4
Wild type	0.4	22.3	20.5	45.1	11.3	0.3
15 F170W+G300W	0.7	21.7	22.5	46.7	8.0	0.3

These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in F170W+G300W.

20 Example D6: Construction and production of variant F170W+G300W+H313R

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

Site specific mutations were induced in the same manner as in Example D1 except that the DNA primers of SEQ ID Nos. 28, 29 and 32 as shown in the sequence listing were used to construct plasmid pAN120 (F170W+G300W+H313R).
25

The result of sequencing for the inserted fragment of pAN120 (F170W+G300W+H313R) confirmed that substitution occurred only in the target nucleotides and no other part of the sequence. In other
30 words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that tryptophan was substituted for phenylalanine at position 170 and glycine at position 300, and arginine for histidine at position 313.

(2) Production of variant F170W+G300W+H313R by yeast
35 A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (F170W+G300W+H313R) by digesting it with BamHI, and inserted into the BamHI site of pY2831

(plasmid pYSUC (F170W+G300W+H313R)).

Plasmid pYSUC (F170W+G300W+H313R) was introduced in the yeast *Saccharomyces cerevisiae* MS-161 in the same manner as in Example D1 to produce variant F170W+G300W+H313R. The culture supernatant exhibited a β -fructofuranosidase activity of 0.9 units/ml.

(3) Evaluation of variant F170W+G300W+H313R

The wild type enzyme and the variant F170W+G300W+H313R were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 °C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and the variant when 1-kestose (GF2) yield was at maximum were as follows:

	F	G	GF	GF2	GF3	GF4
Wild type	0.4	22.3	20.5	45.1	11.3	0.3
F170W+G300W+H313R	1.4	24.0	18.6	48.8	7.2	0.0

These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in F170W+G300W+H313R.

Example D7: Construction and production of variant G300W+H313K

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

Site specific mutations were induced in the same manner as in Example D1 except that the DNA primers of SEQ ID Nos. 29 and 30 as shown in the sequence listing were used to construct plasmid pAN120 (G300W+H313K).

The result of sequencing for the inserted fragment of pAN120 (G300W+H313K) confirmed that substitution occurred only in the target nucleotides and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that tryptophan was substituted for glycine at position 300, and lysine for histidine at position 313.

(2) Production of variant G300W+H313K by yeast

A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (G300W+H313K) by digesting it with

BamHI, and inserted into the BamHI site of pY2831 (plasmid pYSUC (G300W+H313K)).

- Plasmid pYSUC (G300W+H313K) was introduced in the yeast *Saccharomyces cerevisiae* MS-161 in the same manner as in Example D1 to produce variant G300W+H313K. The culture supernatant exhibited a β -fructofuranosidase activity of 1.2 units/ml.

(3) Evaluation of variant G300W+H313K

- The wild type enzyme and the variant G300W+H313K were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 °C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and the variant when 1-kestose (GF2) yield was at maximum were as follows:

	F	G	GF	GF2	GF3	GF4
Wild type	0.4	22.3	20.5	45.1	11.3	0.3
C300W+H313K	0.8	21.2	19.4	53.8	4.7	0.0

- These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in G300W+H313K.

Example D8: Construction and production of variant G300V+H313K

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

- Site specific mutations were induced in the same manner as in Example D1 except that the DNA primers of SEQ ID Nos. 30 and 33 as shown in the sequence listing were used to construct plasmid pAN120 (G300V+H313K).

- The result of sequencing for the inserted fragment of pAN120 (G300V+H313K) confirmed that substitution occurred only in the target nucleotides and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that valine was substituted for glycine at position 300, and lysine for histidine at position 313.

(2) Production of variant G300V+H313K by yeast

A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (G300V+H313K) by digesting it with

BamHI, and inserted into the BamHI site of pY2831 (plasmid pYSUC (G300V+H313K)).

- Plasmid pYSUC (G300V+H313K) was introduced in the yeast *Saccharomyces cerevisiae* MS-161 in the same manner as in Example D1 to produce variant G300V+H313K. The culture supernatant exhibited a β -fructofuranosidase activity of 3.6 units/ml.

(3) Evaluation of variant G300V+H313K

- The wild type enzyme and the variant G300V+H313K were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 °C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and the variant when 1-kestose (GF2) yield was at maximum were as follows:

	F	G	GF	GF2	GF3	GF4
Wild type	0.4	22.3	20.5	45.1	11.3	0.3
G300V+H313K	0.9	21.6	19.0	53.7	4.7	0.0

- These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in G300V+H313K.

Example D9: Construction and production of variant G300E+H313K

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

- Site specific mutations were induced in the same manner as in Example D1 except that the DNA primers of SEQ ID Nos. 30 and 34 as shown in the sequence listing were used to construct plasmid pAN120 (G300E+H313K).

- The result of sequencing for the inserted fragment of pAN120 (G300E+H313K) confirmed that substitution occurred only in the target nucleotides and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that glutamic acid was substituted for glycine at position 300, and lysine for histidine at position 313.

- (2) Production of variant G300E+H313K by yeast

A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (G300E+H313K) by digesting it with

BamHI, and inserted into the BamHI site of pY2831 (plasmid pYSUC (G300E+H313K)).

- 5 Plasmid pYSUC (G300E+H313K) was introduced in the yeast *Saccharomyces cerevisiae* MS-161 in the same manner as in Example D1 to produce variant G300E+H313K. The culture supernatant exhibited a β -fructofuranosidase activity of 2.9 units/ml.

(3) Evaluation of variant G300E+H313K

- 10 The wild type enzyme and the variant G300E+H313K were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 °C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and the variant when 1-kestose (GF2) yield was at maximum were as follows:

15		F	G	GF	GF2	GF3	GF4
	Wild type	0.4	22.3	20.5	45.1	11.3	0.3
	G300E+H313K	1.2	22.0	19.3	52.8	4.7	0.0

- 20 These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in G300E+H313K.

Example D10: Construction and production of variant G300D+H313K

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

- 25 Site specific mutations were induced in the same manner as in Example D1 except that the DNA primers of SEQ ID Nos. 30 and 35 as shown in the sequence listing were used to construct plasmid pAN120 (G300D+H313K).

- 30 The result of sequencing for the inserted fragment of pAN120 (G300D+H313K) confirmed that substitution occurred only in the target nucleotides and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that aspartic acid was substituted for glycine at position 300, and lysine for histidine at position 313.

- 35 (2) Production of variant G300D+H313K by yeast

A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (G300D+H313K) by digesting it with

BamHI, and inserted into the BamHI site of pY2831 (plasmid pYSUC (G300D+H313K)).

Plasmid pYSUC (G300D+H313K) was introduced in the yeast *Saccharomyces cerevisiae* MS-161 in the same manner as in Example D1 to produce variant G300D+H313K. The culture supernatant exhibited a β -fructofuranosidase activity of 4.3 units/ml.

(3) Evaluation of variant G300D+H313K

The wild type enzyme and the variant G300D+H313K were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 °C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and the variant when 1-kestose (GF2) yield was at maximum were as follows:

	F	G	GF	GF2	GF3	GF4
Wild type	0.4	22.3	20.5	45.1	11.3	0.3
G300D+H313K	0.5	21.6	19.6	53.3	5.0	0.0

These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in G300D+H313K.

Example D11: Construction and production of variant F170W+G300W+H313K

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation Site specific mutations were induced in the same manner as in Example D1 except that the DNA primers of SEQ ID Nos. 28, 29 and 30 as shown in the sequence listing were used to construct plasmid pAN120 (F170W+G300W+H313K).

The result of sequencing for the inserted fragment of pAN120 (F170W+G300W+H313K) confirmed that substitution occurred only in the target nucleotides and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that tryptophan was substituted for phenylalanine at position 170 and glycine at position 300, and lysine for histidine at position 313.

(2) Production of variant F170W+G300W+H313K by yeast

A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (F170W+G300W+H313K) by

digesting it with BamHI, and inserted into the BamHI site of pY2831 (plasmid pYSUC (F170W+G300W+H313K)).

Plasmid pYSUC (F170W+G300W+H313K) was introduced in the yeast *Saccharomyces cerevisiae* MS-161 in the same manner as in Example D1 to produce variant F170W+G300W+H313K. The culture supernatant exhibited a β -fructofuranosidase activity of 2.0 units/ml.

(3) Evaluation of variant F170W+G300W+H313K

The wild type enzyme and the variant F170W+G300W+H313K were evaluated using the yeast culture supernatant. After reaction in a 48 wt% sucrose solution, pH 7, at 40 °C, the sugar composition was analyzed by HPLC. The sugar compositions (%) for the wild type and the variant when 1-kestose (GF2) yield was at maximum were as follows:

	F	G	GF	GF2	GF3	GF4
Wild type	0.4	22.3	20.5	45.1	11.3	0.3
F170W+G300W+H313K	0.7	22.3	18.9	54.3	3.9	0.0

These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in F170W+G300W+H313K.

(4) Production of variant F170W+G300W+H313K by *Aspergillus niger* and its evaluation

A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (F170W+G300W+H313K) by digesting it with BamHI, and inserted into the BamHI site of pAN205 (see Example C5) as shown in Figure 9 (plasmid pAN531).

Plasmid pAN531 was digested with HindIII to linearize, then used to transform the *Aspergillus niger* NIA1602 (Suc⁻, niaD⁻). The chromosomal DNA of the transformant was subjected to the Southern analysis, in order to select transformant in which only one copy of plasmid pAN531 was inserted at the location of β -fructofuranosidase gene on the host chromosome by homologous recombination in the promoter region of the β -fructofuranosidase gene.

Next, to delete the vector DNA from the transformant, conidia were prepared and applied to a medium containing chlorate (6% potassium chlorate, 3% sucrose, 0.2% sodium glutamate, 0.1%

K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.01% FeSO₄·7H₂O and 1.5% agar). It was assumed that a transformant which formed colonies on the medium had lost the vector DNA as a result of a secondary homologous recombination. If the secondary recombination took place in the same promoter region as in the first one, the transformant would change to the original host; it took place in the terminator region of the β -fructofuranosidase gene, the gene encoding the F170W+G300W+H313K variant would remain. These two types of recombinants would easily be distinguished by β -fructofuranosidase activity. In the experiment, the ratio between chlorate-resistant strains with β -fructofuranosidase activity and those without was 1:1. The result of Southern analysis for the chromosomal DNA extracted from one of the variants which exhibited β -fructofuranosidase activity, named *Aspergillus niger* NIA3144 (Suc⁺, niaD), confirmed that the vector DNA was missing and the gene encoding the F170W+G300W+H313K variant was inserted at the location of the β -fructofuranosidase gene on the host chromosome.

Next, the *Aspergillus niger* NIA3144 was cultivated in an enzyme production medium (5% sucrose, 0.7% malt extract, 1% polypepton, 0.5% carboxymethyl cellulose and 0.3% NaCl) at 28 °C for 3 days. After the mycelia were ultrasonically homogenized, the β -fructofuranosidase activity of the homogenate was measured. The activity was 25 units per 1 ml of culture solution. The homogenate was added to a 55 wt% sucrose solution, pH 7, at a rate of 2.5 units per 1 g of sucrose, and maintained at 40 °C for 20 hours. After the reaction, the sugar composition as measured by HPLC was 1.2% fructose, 22.8% glucose, 17.1% sucrose, 55.3% GF2 and 3.8% GF3.

(5) Preparation and enzymology of variant F170W+G300W+H313K

The homogenate prepared in (4) above was dialyzed with 20 mM Tris-HCl (pH 7.5) buffer solution, then subjected to a DEAE Toyopearl 650S (Tosoh) column (1.6 × 18 cm), which had been equalized with the same buffer solution, and eluted in Tris-HCl (pH 7.5) buffer solution with a linear gradient of 0 to 300 mM NaCl concentration. The collected active fraction was subjected to (applied to) a Sephacryl S-300 (Pharmacia) column (2.6 × 60 cm), and eluted in 50 mM

trimethylamine-acetate buffer solution (pH 8.0). The collected active fraction was used as a purified F170W+G300V+H313K variant sample. As a result of SDS-polyacrylamide gel electrophoresis, the sample exhibited a single band at about 100,000 Da as did the original β -fructofuranosidase.

Further, the optimum pH, optimum temperature, stability to pH, and stability to temperature of the purified sample were almost the same as those of the original β -fructofuranosidase.

Example D12: Construction and production of variant F170W+G300V+H313K

(1) Nucleotide substitution in β -fructofuranosidase gene by site-specific mutation

Site specific mutations were induced in the same manner as in Example D1 except that the DNA primers of SEQ ID Nos. 28, 30 and 33 as shown in the sequence listing were used to construct plasmid pAN120 (F170W+G300V+H313K).

The result of sequencing for the inserted fragment of pAN120 (F170W+G300V+H313K) confirmed that substitution occurred only in the target nucleotides and no other part of the sequence. In other words, the β -fructofuranosidase encoded by the variant gene was the same as the original enzyme except that tryptophan was substituted for phenylalanine at position 170, valine for glycine at position 300, and lysine for histidine at position 313.

(2) Production of variant F170W+G300V+H313K by *Aspergillus niger* and its evaluation

A 2 kbp BamHI DNA fragment including the variant gene was prepared from plasmid pAN120 (F170W+G300V+H313K) by digesting it with BamHI, and inserted into the BamHI site of pAN205 (plasmid pAN517).

Plasmid pAN517 was digested with HindIII to linearize, then used to transform the *Aspergillus niger* NIA1602 (Suc⁻, niaD) to prepare the *Aspergillus niger* NIA1717 (Suc⁺, niaD), in which the vector DNA was missing and the gene encoding the F170W+G300V+H313K variant was inserted at the location of the β -fructofuranosidase gene on the host chromosome, in the same manner as in Example D11.

Next, the *Aspergillus niger* NIA1717 was cultivated in an

enzyme production medium (5% sucrose, 0.7% malt extract, 1% polypepton, 0.5% carboxymethyl cellulose and 0.3% NaCl) at 28 °C for 3 days. After the mycelia were ultrasonically homogenized, the β -fructofuranosidase activity of the homogenate was measured.

- 5 The activity was 45 units per 1 ml of culture solution. The homogenate was added to a sucrose solution, Bx 45, pH 7.5, at a rate of 2.5 units per 1 g of sucrose, and maintained reaction at 40 °C for 24 hours. After the reaction, the sugar composition as measured by HPLC was 1.8% fructose, 22.3% glucose, 16.1% sucrose, 55.7% GF2 and 4.1% GF3. These figures indicate that GF2 increases and GF3 decreases as a result of the substitution in F170W+G300V+H313K.

(3) Preparation and enzymology of variant F170W+G300V+H313K

- The homogenate prepared in (2) above was dialyzed with 20 mM Tris-HCl (pH 7.5) buffer solution, then subjected to (applied to) a DEAE Toyopearl 650S (Tosoh) column (1.6 × 18 cm), which had been equalized with the same buffer solution, and eluted in Tris-HCl (pH 7.5) buffer solution with a linear gradient of 0 to 300 mM NaCl concentration. The collected active fraction was subjected to (applied to) a Sephacryl S-300 (Pharmacia) column (2.6 × 60 cm), and eluted in 50 mM trimethylamine-acetate buffer solution (pH 8.0). The collected active fraction was used as a purified F170W+G300V+H313K variant sample. As a result of SDS-polyacrylamide gel electrophoresis, the sample exhibited a single band at about 100,000 Da as did the original β -fructofuranosidase.

Further, the optimum pH, optimum temperature, stability to pH, and stability to temperature of the purified sample were almost the same as those of the original β -fructofuranosidase.

Sequence Listing

SEQ ID No. 1

Length: 635

5 Type: amino acid

Molecule type: protein

Source

Microorganism: *Aspergillus niger* ACE-2-1 (ATCC 20611)

Feature of sequence

10 Feature key: mat peptide

Location: 1..635

Identification method: E

Sequence

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            35             40             45
20  Pro Ser Thr Gly Leu Phe His Val Gly Phe Leu His Asp Gly Asp Gly
      50             55             60
      Ile Ala Gly Ala Thr Thr Ala Asn Leu Ala Thr Tyr Thr Asp Thr Ser
      65             70             75             80
      Asp Asn Gly Ser Phe Leu Ile Gln Pro Gly Gly Lys Asn Asp Pro Val
25  Ala Val Phe Asp Gly Ala Val Ile Pro Val Gly Val Asn Asn Thr Pro
      85             90             95
      Ala Val Phe Asp Gly Ala Val Ile Pro Val Gly Val Asn Asn Thr Pro
            100            105            110
      Thr Leu Leu Tyr Thr Ser Val Ser Phe Leu Pro Ile His Trp Ser Ile
            115            120            125
30  Pro Tyr Thr Arg Gly Ser Glu Thr Gln Ser Leu Ala Val Ala Arg Asp
      130            135            140
      Gly Gly Arg Arg Phe Asp Lys Leu Asp Gln Gly Pro Val Ile Ala Asp
      145            150            155            160
      His Pro Phe Ala Val Asp Val Thr Ala Phe Arg Asp Pro Phe Val Phe
35  Ala Val Phe Ala Val Asp Val Thr Ala Phe Arg Asp Pro Phe Val Phe
      165            170            175
      Arg Ser Ala Lys Leu Asp Val Leu Leu Ser Leu Asp Glu Glu Val Ala
            180            185            190

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	Arg	Asn	Glu	Thr	Ala	Val	Gln	Gln	Ala	Val	Asp	Gly	Trp	Thr	Glu	Lys
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	Asn	Ala	Pro	Trp	Tyr	Val	Ala	Val	Ser	Gly	Gly	Val	His	Gly	Val	Gly
	210						215					220				
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	225					230					235				240	
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				245					250					255		
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	290					295					300					
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	305				310					315					320	
	Glu	Val	Gly	Val	Gly	Ser	Glu	Gln	Glu	Gly	Ala	Lys	Val	Glu	Phe	Ser
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	Ala	Arg	Glu	Thr	Lys	Ala	Ala	Leu	Leu	Ala	Asn	Gly	Ser	Val	Thr	Ala
	450				455					460						
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	465			470						475					480	

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 Ala Ser Ala Arg Ser Ser Pro Leu Gln Ser Gly Phe Glu Ile Leu Ala
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 5 Ser Glu Leu Glu Arg Thr Ala Ile Tyr Tyr Gln Phe Ser Asn Glu Ser
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 Gly Leu Asp Ser Phe Thr Glu Ser Gly Lys Leu Arg Leu Phe Asp Val
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 Ile Glu Asn Gly Gln Glu Gln Val Glu Thr Leu Asp Leu Thr Val Val
 565 570 575
 Val Asp Asn Ala Val Val Glu Val Tyr Ala Asn Gly Arg Phe Ala Leu
 580 585 590
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 Phe His Asn Gly Glu Gly Glu Val Gln Phe Arg Asn Val Ser Val Ser
 610 615 620
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 20 625 630 635

SEQ ID No. 2

Length: 1905

Type: Nucleic acid

25 Strandedness: Double strand

Topology: Linear

Molecule type: Genomic DNA

Source

Microorganism: *Aspergillus niger* ACE-2-1 (ATCC 20611)

30 Feature of sequence

Feature key: mat peptide

Location: 1.. 1905

Identification method: E

Sequence

35 TCATACCACC TGGACACCAC GGGCCCGCCG CCGACCAACC TCAGCACCT CCCCAACAAC 60
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 GACCCCTGCG CGCACTACAC CGACCCATCC ACCGGCTTCT TCCACGTGGG GTTCTGCAC 180

	GACGGGGACG	GCATCGGGG	CGCCACCAG	GCCAACCTGG	CCACCTACAC	CGATACCTCC	240
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	GGGGCCGTCA	TCCCGGTGG	CGTCAACAAC	ACCCCACCT	TACTCTACAC	CTCCGTCTCC	360
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5	GTCCGGCGCG	ACGGCGGCG	CCGCTTCGAC	AAGCTCGACC	AGGGCCCCGT	CATCGCGGAC	480
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	TTGGATGTGC	TGCTGTGTT	GGATGAGGAG	GTGGCGCGGA	ATGAGACGG	CTGACGACG	600
	GCCGTGATG	GCTGGACGA	GAAGAAGCC	CCCTGGTAT	TCCCGGTCT	TGGCGGGTG	660
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	ACGGGCGACC	AGTACGACGA	GCGGACGGG	TTCCCACGG	CCCAGCAGG	GTGGACGGG	1200
	TGCTGCTGCG	TGCCGCGCGA	GCTGAAGGTG	CAGACGGTGG	AGAACGTGCT	GCACAACGAG	1260
	CTGTGCGCG	AGGAGGGCGT	GTCGTGGTG	GTGGGGGAGT	CGGACAACCA	GACGGCCAGG	1320
20	CTGCGCACGC	TGGGGATCAC	GATCGCCCG	GAGACCAAG	CGGCCCTGCT	GGCCAACGGC	1380
	TGCGTGACCG	CGGAGGAGGA	CCGCACGCTG	CAGACGGCG	CCGTCTGTCC	GTCGCGCAA	1440
	TGCGCGAGCT	CAAAGTTCTT	CGTCTGACG	GCCAGCTGG	AGTTCCCCGC	GAGCGCGCG	1500
	TGCTCCCGC	TCCAGTCCGG	GTTGAAATC	CTGGGTCGG	AGCTGGAGCG	CACGGCCATC	1560
	TACTACCAGT	TCAGCAACGA	GTCGTGGTC	GTCGACCGCA	GCCAGACTAG	TGCGGCGGCG	1620
25	CCCACGAACC	CCGGGCTGGA	TAGCTTTACT	GAGTCCGGCA	AGTTGCGGTT	GTTGACGCTG	1680
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30

SEQ ID No. 3

Length: 20

Type: amino acid

Topology: Linear

35

Molecule type: peptide

Fragment type: internal fragment

Source

Microorganism: *Aspergillus niger* ACE-2-1 (ATCC 20611)

Sequence

Leu Asp Gln Gly Pro Val Ile Ala Asp His Pro Phe Ala Val Asp Val

1 5 10 15

5 Thr Ala Phe Arg

20

SEQ ID No. 4

Length: 20

10 Type: amino acid

Topology: Linear

Molecule type: peptide

Fragment type: internal fragment

Source

15 Microorganism: *Aspergillus niger* ACE-2-1 (ATCC 20611)

Sequence

Val Glu Phe Ser Pro Ser Met Ala Gly Phe Leu Asp Trp Gly Phe Ser

1 5 10 15

Ala Tyr Ala Ala

20 20

SEQ ID No. 5

Length: 20

Type: amino acid

25 Topology: Linear

Molecule type: peptide

Fragment type: internal fragment

Source

Microorganism: *Aspergillus niger* ACE-2-1 (ATCC 20611)

30 Sequence

Val Gln Thr Val Glu Asn Val Val Asp Asn Glu Leu Val Arg Glu Glu

1 5 10 15

Gly Val Ser Trp

20

35

SEQ ID No. 6

Length: 20

Type: amino acid
 Topology: Linear
 Molecule type: peptide
 Fragment type: internal fragment

5 Source

Microorganism: *Aspergillus niger* ACE-2-1 (ATCC 20611)

Sequence

Ala Ala Leu Leu Ala Xaa Gly Ser Val Thr Ala Glu Glu Asp Arg Thr

1

5

10

15

10 Leu Gln Thr Ala

20

SEQ ID No. 7

Length: 6

15 Type: amino acid

Topology: Linear

Molecule type: peptide

Fragment type: N-terminal fragment

Source

20 Microorganism: *Aspergillus niger* ACE-2-1 (ATCC 20611)

Sequence

Ser Tyr His Leu Asp Thr

1

5

25 SEQ ID No. 8

Length: 20

Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

30 Sequence

ATCGCSGAYC AYCCSTTYGC 20

SEQ ID No. 9

Length: 20

35 Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

Sequence

TCRTTRTCSA CSACRTTYTC 20

SEQ ID No.10

5 Length: 788

Type: Nucleic acid

Strandedness: Double strand

Topology: Linear

Source

10 Microorganism: *Aspergillus niger* ACE-2-1 (ATCC 20611)

Feature of sequence

Feature key: P CDS(partial amino acid sequence)

Location: 1..788

Identification method: E

15 Sequence

ATC GCC GAC CAC CCC TTC GCC GTC GAC GTC ACC GCC TTC CGC GAT CCG 48

Ile Ala Asp His Pro Phe Ala Val Asp Val Thr Ala Phe Arg Asp Pro

1

5

10

15

TTT GTC TTC CGC AGT GCC AAG TTG GAT GTG CTG CTG TCG TTG GAT GAG 96

20 Phe Val Phe Arg Ser Ala Lys Leu Asp Val Leu Leu Ser Leu Asp Glu

20

25

30

GAG GTG GCG CGG AAT GAG ACG GCC GTG CAG CAG GCC GTC GAT GGC TGG 144

Glu Val Ala Arg Asn Glu Thr Ala Val Gln Gln Ala Val Asp Gly Trp

35

40

45

25 ACC GAG AAG AAC GCC CCC TGG TAT GTC GCG GTC TCT GGC GGG GTG CAC 192

Thr Glu Lys Asn Ala Pro Trp Tyr Val Ala Val Ser Gly Gly Val His

50

55

60

GGC GTC GGG CCC GCG CAG TTC CTC TAC CGC CAG AAC GGC GGG AAC GCT 240

Gly Val Gly Pro Ala Gln Phe Leu Tyr Arg Asn Gly Gly Asn Ala

30 65 70 75 80

TCC GAG TTC CAG TAC TGG GAG TAC CTC GGG GAG TGG TGG CAG GAG GCG 288

Ser Glu Phe Gln Tyr Trp Glu Tyr Leu Gly Glu Trp Trp Gln Glu Ala

85

90

95

ACC AAC TCC AGC TGG GGC GAC GAG GGC ACC TGG GCC GGG CGC TGG GGG 336

35 Thr Asn Ser Ser Trp Gly Asp Glu Gly Thr Trp Ala Gly Arg Trp Gly

100

105

110

	TTC AAC TTC GAG ACG GGG AAT GTG CTC TTC CTC ACC GAG GAG GGC CAT	384
	Phe Asn Phe Glu Thr Gly Asn Val Leu Phe Leu Thr Glu Glu Gly His	
	115 120 125	
	GAC CCC CAG ACG GGC GAG GTG TTC GTC ACC CTC GGC ACG GAG GGG TCT	432
5	Asp Pro Gln Thr Gly Glu Val Phe Val Thr Leu Gly Thr Glu Gly Ser	
	130 135 140	
	GGC CTG CCA ATC GTG CCG CAG GTC TCC AGT ATC CAC GAT ATG CTG TGG	480
	Gly Leu Pro Ile Val Pro Gln Val Ser Ser Ile His Asp Met Leu Trp	
	145 150 155 160	
10	GCG GCG GGT GAG GTC GGG GTG GGC AGT GAG CAG GAG GGT GCC AAG GTC	528
	Ala Ala Gly Glu Val Gly Val Gly Ser Glu Gln Glu Gly Ala Lys Val	
	165 170 175	
	GAG TTC TCC CCC TCC ATG GCC GGG TTT CTG GAC TGG GGG TTC AGC GCC	576
	Glu Phe Ser Pro Ser Met Ala Gly Phe Leu Asp Trp Gly Phe Ser Ala	
15	180 185 190	
	TAC GCT GCG GCG GGC AAG GTG CTG CCG GCC AGC TCG GCG GTG TCG AAG	624
	Tyr Ala Ala Ala Gly Lys Val Leu Pro Ala Ser Ser Ala Val Ser Lys	
	195 200 205	
	ACC AGC GGC GTG GAG GTG GAT CGG TAT GTC TCG TTC GTC TGG TTG ACG	672
20	Thr Ser Gly Val Glu Val Asp Arg Tyr Val Ser Phe Val Trp Leu Thr	
	210 215 220	
	GGC GAC CAG TAC GAG CAG GCG GAC GGG TTC CCC ACG GCC CAG CAG GGG	720
	Gly Asp Gln Tyr Glu Gln Ala Asp Gly Phe Pro Thr Ala Gln Gln Gly	
	225 230 235 240	
25	TGG ACG GGG TCG CTG CTG CTG CCG CGC GAG CTG AAG GTG CAG ACG GTG	768
	Trp Thr Gly Ser Leu Leu Leu Pro Arg Glu Leu Lys Val Gln Thr Val	
	245 250 255	
	GAG AAC GTC GTC GAC AAC GA	788
	Glu Asn Val Val Asp Asn	
30	260	

SEQ ID No. 11

Length: 565

Type: amino acid

35 Molecule type: protein

Source

Microorganism: *Penicillium roqueforti* IAM7254

Feature of sequence

Feature key: mal peptide

Location: I..565

Identification method: E

5 Sequence

Val Asp Phe His Thr Pro Ile Asp Tyr Asn Ser Ala Pro Pro Asn Leu
 1 5 10 15
 Ser Thr Leu Ala Asn Ala Ser Leu Phe Lys Thr Trp Arg Pro Arg Ala
 20 25 30
 10 His Leu Leu Pro Pro Ser Gly Asn Ile Gly Asp Pro Cys Gly His Tyr
 35 40 45
 Thr Asp Pro Lys Thr Gly Leu Phe His Val Gly Trp Leu Tyr Ser Gly
 50 55 60
 Ile Ser Gly Ala Thr Thr Asp Asp Leu Val Thr Tyr Lys Asp Leu Asn
 65 70 75 80
 15 Pro Asp Gly Ala Pro Ser Ile Val Ala Gly Gly Lys Asn Asp Pro Leu
 85 90 95
 Ser Val Phe Asp Gly Ser Val Ile Pro Ser Gly Ile Asp Gly Met Pro
 100 105 110
 20 Thr Leu Leu Tyr Thr Ser Val Ser Tyr Leu Pro Ile His Trp Ser Ile
 115 120 125
 Pro Tyr Thr Arg Gly Ser Glu Thr Gln Ser Leu Ala Val Ser Tyr Asp
 130 135 140
 Gly Gly His Asn Phe Thr Lys Leu Asn Gln Gly Pro Val Ile Pro Thr
 145 150 155 160
 25 Pro Pro Phe Ala Leu Asn Val Thr Ala Phe Arg Asp Pro Tyr Val Phe
 165 170 175
 Gln Ser Pro Ile Leu Asp Lys Ser Val Asn Ser Thr Gln Gly Thr Trp
 180 185 190
 30 Tyr Val Ala Ile Ser Gly Gly Val His Gly Val Gly Pro Cys Gln Phe
 195 200 205
 Leu Tyr Arg Gln Asn Asp Ala Asp Phe Gln Tyr Trp Glu Tyr Leu Gly
 210 215 220
 Gln Trp Trp Lys Glu Pro Leu Asn Thr Thr Trp Gly Lys Gly Asp Trp
 225 230 235 240
 35 Ala Gly Gly Trp Gly Phe Asn Phe Glu Val Gly Asn Val Phe Ser Leu
 245 250 255

	Asn	Ala	Glu	Gly	Tyr	Ser	Glu	Asp	Gly	Glu	Ile	Phe	Ile	Thr	Leu	Gly
							260			265					270	
	Ala	Glu	Gly	Ser	Gly	Leu	Pro	Ile	Val	Pro	Gln	Val	Ser	Ser	Ile	Arg
			275					280					285			
5	Asp	Met	Leu	Trp	Val	Thr	Gly	Asn	Val	Thr	Asn	Asp	Gly	Ser	Val	Thr
		290						295				300				
	Phe	Lys	Pro	Thr	Met	Ala	Gly	Val	Leu	Asp	Trp	Gly	Val	Ser	Ala	Tyr
	305				310					315					320	
	Ala	Ala	Ala	Gly	Lys	Ile	Leu	Pro	Ala	Ser	Ser	Gln	Ala	Ser	Thr	Lys
10				325					330						335	
	Ser	Gly	Ala	Pro	Asp	Arg	Phe	Ile	Ser	Tyr	Val	Trp	Leu	Thr	Gly	Asp
			340						345					350		
	Leu	Phe	Glu	Gln	Val	Lys	Gly	Phe	Pro	Thr	Ala	Gln	Gln	Asn	Trp	Thr
		355						360						365		
15	Gly	Ala	Leu	Leu	Leu	Pro	Arg	Glu	Leu	Asn	Val	Arg	Thr	Ile	Ser	Asn
		370					375					380				
	Val	Val	Asp	Asn	Glu	Leu	Ser	Arg	Glu	Ser	Leu	Thr	Ser	Trp	Arg	Val
	385				390					395					400	
	Ala	Arg	Glu	Asp	Ser	Gly	Gln	Ile	Asp	Leu	Glu	Thr	Met	Gly	Ile	Ser
20					405					410					415	
	Ile	Ser	Arg	Glu	Thr	Tyr	Ser	Ala	Leu	Thr	Ser	Gly	Ser	Ser	Phe	Val
			420						425						430	
	Glu	Ser	Gly	Lys	Thr	Leu	Ser	Asn	Ala	Gly	Ala	Val	Pro	Phe	Asn	Thr
		435							440						445	
25	Ser	Pro	Ser	Ser	Lys	Phe	Phe	Val	Leu	Thr	Ala	Asn	Ile	Ser	Phe	Pro
		450					455						460			
	Thr	Ser	Ala	Arg	Asp	Ser	Gly	Ile	Gln	Ala	Gly	Phe	Gln	Val	Leu	Ser
	465				470					475					480	
	Ser	Ser	Leu	Glu	Ser	Thr	Thr	Ile	Tyr	Tyr	Gln	Phe	Ser	Asn	Glu	Ser
30					485					490					495	
	Ile	Ile	Val	Asp	Arg	Ser	Asn	Thr	Ser	Ala	Ala	Ala	Arg	Thr	Thr	Ala
			500						505						510	
	Gly	Ile	Leu	Ser	Asp	Asn	Glu	Ala	Gly	Arg	Leu	Arg	Leu	Phe	Asp	Val
		515							520						525	
35	Leu	Arg	Asn	Gly	Lys	Glu	Gln	Val	Glu	Thr	Leu	Glu	Leu	Thr	Ile	Val
		530							535						540	

Val Asp Asn Ser Val Leu Glu Val Tyr Ala Asn Gly Arg Phe Ala Leu
 545 550 555 560
 Gly Thr Trp Ala Arg
 565

5

SEQ ID No. 12

Length: 1695

Type: Nucleic acid

Strandedness: Double strand

10 Topology: Linear

Molecule type: Genomic DNA

Source

Microorganism: *Penicillium roqueforti* IAM7254

Feature of sequence

15 Feature key: mal peptide

Location: 1..1695

Identification method: E

Sequence

	GTGATTTC ATACCCGAT TGAATAAC TCGGCTCGC CAAACCTTC TACCCTGGCA	60
20	AACGCATCT TTTTCAAGAC ATGGAGACCC AGAGCCCATC TTCTCCCTCC ATCTGGGAAC	120
	ATAGGCGACC CGTGGGGGCA CTATACCGAT CCCAAGACTG GTCTCTTCCA CGTGGGTGG	180
	CTTTACAGTG GGAATTCGGG AGCGACAACC GACGATCTCG TTACCTATAA AGACCTCAAT	240
	CCCGATGGAG CCCCGTCAAT TGTTCAGGA GGAAGAAGC ACCCTCTTC TGTCTTCGAT	300
	GGCTCGGTCA TTCCAAGCGG TATAGACGGC ATGCCAACTC TTCTGTATAC CTCTGTATCA	360
25	TACCTCCCAA TCCACTGGTC CATCCCCTAC ACCCGGGGAA GCGAGACACA ATCCTTGGCC	420
	GTTTCCTATG ACGGTGGTCA CAACCTCACC AAGCTCAACC AAGGGCCCGT GATCCCTACG	480
	CCTCCGTTTG CTCTCAATGT CACCGCTTTC CGTGACCCCT ACGTTTCCA AAGCCCAATT	540
	CTGGACAAAT CTGTCAATAG TACCCAAGGA ACATGGTATG TCGCCATATC TGGCGGTGTC	600
	CACGGTGTG GACCTTGTC GTTCTCTAC CGTCAGAAGC ACGCAGATTT TCAATATTGG	660
30	GAATATCTCG GGAATGGTG GAAGGAGCCC CTTAATAACA CTGGGGGAAA GGGTGACTGG	720
	GCCGGGGGTT GGGGCTTCAA CTTTGAGGTT GGCACGCTCT TTAGTCTGAA TGCAGAGGGG	780
	TATAGTGAAG ACGGCGAGAT ATTCAATAACC CTCGGTGCTG AGGGTTCGG ACTTCCCATC	840
	GTTCTCAAG TCTCTCTAT TCGCGATATG CTGTGGTGA CCGGCAATGT CACAAATGAC	900
	GGCTCTGTCA CTTTCAAGCC AACCATGGCG GGTGTGCTTG ACTGGGGCGT GTCGGCATAT	960
35	GCTGCTGCAG GCAAGATCTT GCCGGCCAGC TCTCAGGCAT CCACAAAGAG CGGTGCCCCC	1020
	GATCGGTTCA TTTCTATGT CTGGCTCACT GGAGATCTAT TCGAGCAAGT GAAAGGATTC	1080
	CCTACCGCTC AACAAACTG GACCGGGGCC CTCTTACTGC CGCGAGAGCT GAATGTCCGC	1140

ACTATCTCTA ACGTGGTGA TAACGAACCT TCGCGTGAGT CCTTGACATC GTGGCGCGTG 1200
 GCCCGCGAAG ACTCTGGTCA GATCGACCTT GAAACAATGG GAATCTCAAT TTCCAGGGAG 1260
 ACTTACAGCG CTCTCACATC CGGCTCATCT TTTGTCGAGT CTGGTAAAC GTTGTCGAAT 1320
 GCTGGAGCAG TGCCTTCAA TACCTCACC TCAAGCAAGT TCTTCGTGCT GACAGCAAT 1380
 5 ATATCTTTCC CGACCTCTGC CCGTGACTCT GGCATCCAGG CTGGTTTCCA GGTTTTATCC 1440
 TCTAGTCTTG AGTCTACAAC TATCTACTAC CAATTCTCCA ACGAGTCCAT CATCGTCGAC 1500
 CGCAGCAACA CGAGTGTGCG GCGGAGAACA ACTGCTGGGA TCCTCAGTGA TAACGAGGCG 1560
 GGACGCTGCG GCCTCTTCGA CGTGTTCGGA AATGGAAAAG AACAGTTGA AACTTTGGAG 1620
 CTCACTATCG TGGTGGATAA TAGTGTACTG GAAGTATATG CCAATGGACG CTTTGCTCTA 1680
 10 GGCACCTGGG CTCGG 1695

SEQ ID No.13

Length: 574

Type: amino acid

15 Molecule type: protein

Source

Microorganism: *Scopulariopsis brevicaulis* IF04843

Feature of sequence

Feature key: mat peptide

20 Location: 1..574

Identification method: E

Sequence

Gln Pro Thr Ser Leu Ser Ile Asp Asn Ser Thr Tyr Pro Ser Ile Asp

1 5 10 15

25 Tyr Asn Ser Ala Pro Pro Asn Leu Ser Thr Leu Ala Asn Asn Ser Leu

20 25 30

Phe Glu Thr Trp Arg Pro Arg Ala His Val Leu Pro Pro Gln Asn Gln

35 40 45

Ile Gly Asp Pro Cys Met His Tyr Thr Asp Pro Glu Thr Gly Ile Phe

50 55 60

30 His Val Gly Trp Leu Tyr Asn Gly Asn Gly Ala Ser Gly Ala Thr Thr

65 70 75 80

Glu Asp Leu Val Thr Tyr Gln Asp Leu Asn Pro Asp Gly Ala Gln Met

85 90 95

35 Ile Leu Pro Gly Gly Val Asn Asp Pro Ile Ala Val Phe Asp Gly Ala

100 105 110

Val Ile Pro Ser Gly Ile Asp Gly Lys Pro Thr Met Met Tyr Thr Ser
 115 120 125
 Val Ser Tyr Met Pro Ile Ser Trp Ser Ile Ala Tyr Thr Arg Gly Ser
 130 135 140
 5 Glu Thr His Ser Leu Ala Val Ser Ser Asp Gly Gly Lys Asn Phe Thr
 145 150 155 160
 Lys Leu Val Gln Gly Pro Val Ile Pro Ser Pro Pro Phe Gly Ala Asn
 165 170 175
 Val Thr Ser Trp Arg Asp Pro Phe Leu Phe Gln Asn Pro Gln Phe Asp
 180 185 190
 10 Ser Leu Leu Glu Ser Glu Asn Gly Thr Trp Tyr Thr Val Ile Ser Gly
 195 200 205
 Gly Ile His Gly Asp Gly Pro Ser Ala Phe Leu Tyr Arg Gln His Asp
 210 215 220
 15 Pro Asp Phe Gln Tyr Trp Glu Tyr Leu Gly Pro Trp Trp Asn Glu Glu
 225 230 235 240
 Gly Asn Ser Thr Trp Gly Ser Gly Asp Trp Ala Gly Arg Trp Gly Tyr
 245 250 255
 Asn Phe Glu Val Ile Asn Ile Val Gly Leu Asp Asp Asp Gly Tyr Asn
 260 265 270
 20 Pro Asp Gly Glu Ile Phe Ala Thr Val Gly Thr Glu Trp Ser Phe Asp
 275 280 285
 Pro Ile Lys Pro Gln Ala Ser Asp Asn Arg Glu Met Leu Trp Ala Ala
 290 295 300
 25 Gly Asn Met Thr Leu Glu Asp Gly Asp Ile Lys Phe Thr Pro Ser Met
 305 310 315 320
 Ala Gly Tyr Leu Asp Trp Gly Leu Ser Ala Tyr Ala Ala Ala Gly Lys
 325 330 335
 Glu Leu Pro Ala Ser Ser Lys Pro Ser Gln Lys Ser Gly Ala Pro Asp
 340 345 350
 30 Arg Phe Val Ser Tyr Leu Trp Leu Thr Gly Asp Tyr Phe Glu Gly His
 355 360 365
 Asp Phe Pro Thr Pro Gln Gln Asn Trp Thr Gly Ser Leu Leu Leu Pro
 370 375 380
 35 Arg Glu Leu Ser Val Gly Thr Ile Pro Asn Val Val Asp Asn Glu Leu
 385 390 395 400

	Ala Arg Glu Thr Gly Ser Trp Arg Val Gly Thr Asn Asp Thr Gly Val	
	405	410 415
	Leu Glu Leu Val Thr Leu Lys Gln Glu Ile Ala Arg Glu Thr Leu Ala	
	420	425 430
5	Glu Met Thr Ser Gly Asn Ser Phe Thr Glu Ala Ser Arg Asn Val Ser	
	435	440 445
	Ser Pro Gly Ser Thr Ala Phe Gln Gln Ser Leu Asp Ser Lys Phe Phe	
	450	455 460
	Val Leu Thr Ala Ser Leu Ser Phe Pro Ser Ser Ala Arg Asp Ser Asp	
10	465	470 475 480
	Leu Lys Ala Gly Phe Glu Ile Leu Ser Ser Glu Phe Glu Ser Thr Thr	
	485	490 495
	Val Tyr Tyr Gln Phe Ser Asn Glu Ser Ile Ile Ile Asp Arg Ser Asn	
	500	505 510
15	Ser Ser Ala Ala Ala Leu Thr Thr Asp Gly Ile Asp Thr Arg Asn Glu	
	515	520 525
	Phe Gly Lys Met Arg Leu Phe Asp Val Val Glu Gly Asp Gln Glu Arg	
	530	535 540
	Ile Glu Thr Leu Asp Leu Thr Ile Val Val Asp Asn Ser Ile Val Glu	
20	545	550 555 560
	Val His Ala Asn Gly Arg Phe Ala Leu Ser Thr Trp Val Arg	
	565	570

SEQ ID No. 14

25 Length: 1722

Type: Nucleic acid

Strandedness: Double strand

Topology: Linear

Molecule type: Genomic DNA

30 Source

Microorganism: *Scopulariopsis brevicaulis* IF04843

Feature of sequence

Feature key: mal peptide

Location: 1..1722

35 Identification method: E

Sequence

CAACCTACGT CTCTGTCAAT CGACAATTCC ACGTATCCTT CTATCGACTA CAACTCCGCC

60

	CCTCCAAACC	TCTCGACTCT	TGCCAAACAAC	AGCCTCTTGG	AGACATGGAG	GCCGAGGGCA	120
	CACGTCCTTC	CGCCCCAGAA	CCAGATCGGC	GATCCGTGTA	TGCACTACAC	CGACCCCGAG	180
	ACAGGAATCT	TCCACGTCGG	CTGGCTGTAC	AACGGCAATG	GCGCTTCCGG	CGCCACGACC	240
	GAGGATCTCG	TCACCTATCA	GGATCTCAAC	CECGACGGAG	CGCAGATGAT	CCTTCCGGGT	300
5	GGTGTGAATG	ACCCCAATTGC	TGTCTTTGAC	GGCGGGTTA	TTCCCACTGG	CATTGATGGG	360
	AAACCCACCA	TGATGTATAC	CTCGGTGTCA	TACATCCCCA	TCTCTGGAG	CATCGTTTAC	420
	ACCAGGGGAA	GCGAGACCCA	CTCTCTCGCA	GTGTCGTCCG	ACGGCGGTAA	GAATTTCACC	480
	AAGCTGGTGG	AGGGCCCCGT	CATTCTCTCG	CCTCCCTTCG	GCGCCAACGT	GACCAGCTGG	540
	CGTGACCCCT	TCCTGTTCCA	AAACCCCCAG	TTGCACTCTC	TCCTCGAAAG	CGAGAACGGC	600
10	ACGTGGTACA	CCGTTATCTC	TGGTGGCATC	CACGGTGACG	GCCCTCCCG	GTTCTCTTAC	660
	CGTCAGCAGC	ACCCCGACTT	CCAGTACTGG	GAGTACCTTG	GACCGTGGTG	GAACGAGGAA	720
	GGGAACTCGA	CCTGGGGCAG	CGGTGACTGG	GCTGGCCGGT	GGGGCTACAA	CTTCGAGGTC	780
	ATCAACATTG	TCGGCTTTGA	CGATGATGGC	TACAACCCCG	ACGGTGAAT	CTTGGCCACG	840
	GTAGGTACCG	AATGGTGGTT	TGACCCCATC	AAACCGCAGG	CCTCGGACAA	CAGGAGATG	900
15	CTCTGGGCGG	CGGGCAACAT	GACTCTCGAG	GACGGCGATA	TCAAGTTTAC	GCCAAGCATG	960
	GCGGGCTACC	TCCACTGGGG	TCTATCGGCG	TATGCCGCCG	TGGCAAGGA	GCTGCCCGCT	1020
	TCTTCAAAGC	CTTCGAGAA	GAGCGGTGCG	CCGGACCGGT	TCGTGTGCTA	CCTGTGGGTC	1080
	ACCGGTGACT	ACTTCGAGGG	CCACGACTTC	CCCACCCCG	AGCAGAATTG	GACCGGCTCG	1140
	CTTTTGCTTC	CGCGTGAGCT	GAGCGTCGGG	ACGATTCCCA	ACGTTGTGCA	CAACGAGCTT	1200
20	GCTCGGAGAG	CGGGCTCTTG	GAGGGTTGGC	ACCAACGACA	CTGGCGTGCT	TGAGCTGGTC	1260
	ACTCTGAAGC	AGGAGATTGC	TCGGGAGACG	CTGGCTGAAA	TGACCAGCGG	CAACTCTTTC	1320
	ACCGAGGCGA	GCAGGAATGT	CAGCTCGCCC	GGATCTACCG	CCTTCCAGCA	GTCCCTGGAT	1380
	TCCAAGTTCT	TCGTCTTGAC	CGCCTCGCTC	TCCTTCCCTT	CGTCGGCTCG	GCACTCCGAC	1440
	CTCAAGGCTG	GTTTCGAGAT	CCTGTCGTCC	GAGTTTGAGT	CGACCACGGT	CTACTACCAG	1500
25	TTTTCCAACG	AGTCCATCAT	CATTGACCGG	AGCAACTCGA	GTGCTGCCGC	CTTGACTACC	1560
	GATGGAATCG	ACACCCGCAA	CGAGTTTGGC	AAGATGCGCC	TGTTTGATGT	TGTCGAGGGT	1620
	GACCAGGAGC	GTATCGAGAC	GCTCGATCTC	ACTATTGTGG	TGTGAACCTC	GATCGTTGAG	1680
	GTTCATGCCA	ACGGGCGATT	CGCTCTGAGC	ACTTGGGTTG	GG		1722

30 SEQ ID No. 15

Length: 28

Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

35 Sequence

CGGAATTCCA ATGAAGTCA CCACTACC 28

SEQ ID No. 16

Length: 24

Type: Nucleic acid

Topology: Linear

5 Molecule type: Synthetic DNA

Sequence

GCGGATCCCG GTCAATTCT CTCC 24

SEQ ID No. 17

10 Length: 19

Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

Sequence

15 GACTGACCGG TGTTCATCC

SEQ ID No. 18

Length: 20

Type: Nucleic acid

20 Topology: Linear

Molecule type: Synthetic DNA

Sequence

CTCGGTTGTC ATAGATGTGG

25 SEQ ID No. 19

Length: 24

Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

30 Sequence

CAATCCAGGA GGATCCCAAT GAAG

SEQ ID No. 20

Length: 22

35 Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

Sequence

TGACCGGAT CCGGCATGC AG

SEQ ID No. 21

5 Length: 24

Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

Sequence

10 CGCGTCGTCT AGAGGTGTC ACTT

SEQ ID No. 22

Length: 21

Type: Nucleic acid

15 Topology: Linear

Molecule type: Synthetic DNA

Sequence

CCCTATTGGG GTCCATGGCC C

20 SEQ ID No. 23

Length: 22

Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

25 Sequence

CAACTGCTGG CATCCTCAGT GA

SEQ ID No. 24

30 Length: 30

Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

Sequence

35 GCGGATCCAT GAAGCTATCA AATGCAATCA

SEQ ID No. 25

Length: 26
Type: Nucleic acid
Topology: Linear
Molecule type: Synthetic DNA

5 Sequence
GCGGATCCTT ACCGAGCCCA AGTGCC

SEQ ID No. 26

Length: 27

10 Type: Nucleic acid
Topology: Linear
Molecule type: Synthetic DNA
Sequence

GCGGATCCAA TGAAGCTCAC CACTACC

15 0

SEQ ID No. 27

Length: 24

Type: Nucleic acid
Topology: Linear

20 Molecule type: Synthetic DNA
Sequence

GCGGATCCCG GTCAATTCT CTCC

SEQ ID No. 28

25 Length: 21

Type: Nucleic acid
Topology: Linear
Molecule type: Synthetic DNA
Sequence

30 GTCACCGCCT GCGCGATCC G

SEQ ID No. 29

Length: 19

Type: Nucleic acid
35 Topology: Linear
Molecule type: Synthetic DNA
Sequence

GGCACGGAGT GGTCTGGCC

SEQ ID No. 30

Length: 24

5 Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

Sequence

CTCCAGTATC AAGGATATGC TGTG

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SEQ ID No. 31

Length: 20

Type: Nucleic acid

Topology: Linear

15 Molecule type: Synthetic DNA

Sequence

CGACCACTAC AAGCAGGCGG

SEQ ID No. 32

20 Length: 21

Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

Sequence

25 TCCAGTATCC GCGATATGCT G

SEQ ID No. 33

Length: 23

Type: Nucleic acid

30 Topology: Linear

Molecule type: Synthetic DNA

Sequence

CGGCACGGAG GTTCTGGCC TGC

35 SEQ ID No. 34

Length: 23

Type: Nucleic acid

Topology: Linear

Molecule type: Synthetic DNA

Sequence

CGGCACGGAG GAGTCTGGCC TGC

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SEQ ID No. 35

Length: 23

Type: Nucleic acid

Topology: Linear

10 Molecule type: Synthetic DNA

Sequence

CGGCACGGAG GATTCTGGCC TGC

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